THE INFLUENCE OF ETHANOL ON THE BIOTRANSFORMATION AND GENOTOXICITY OF BENZO(*A*)PYRENE

JEANINE A.G. VAN DE WIEL

THE INFLUENCE OF ETHANOL ON THE BIOTRANSFORMATION AND GENOTOXICITY OF BENZO(*A*)PYRENE

1. EXPOSURE TO XENOBIOTICS

1.1 Exposure to xenobiotics and relation to cancer

People living in the industrialized countries are continually exposed to a large number of different chemicals. Many of such chemicals cause acute effects, but long-term exposures are those that are most interesting in terms of potential delayed consequences, such as cancer (Pelkonen & Sotaniemi EA, 1987). In the actual situation, at least in the Netherlands, one out of four people dies from this disease (Centraal Bureau voor de Statistiek, 1980).

Epidemiologists have estimated that at least 70% of human cancer would, in principle, be preventable if the main risk and antirisk factors could be identified (Doll & Peto, 1981). This is because the incidence of specific types of cancer differs markedly in different parts of the world where people have different lifestyles. Colon and breast cancer, which are among the major types of cancer in the United States, are quite rare among Japanese in Japan, but not among Japanese Americans. Epidemiologists are providing important clues about the specific causes of human cancer, in spite of inherent methodological difficulties. They have identified tobacco as an avoidable cause of about 30% of all U.S. cancer deaths. Less specifically, dietary factors, or the absence of certain dietary components, have been suggested in many studies to contribute to a substantial proportion of cancer deaths, though the intertwined risk and antirisk factors are being identified only slowly (Hopkins & Carroll, 1985; Bird *et al.*, 1986). High fat intake may be a major contributor to colon cancer, though the evidence is not as definitive as that for the role of saturated fat in heart disease or of tobacco smoke in lung cancer. Alcoholic beverage consumption, particularly by smokers, has been estimated to contribute to about 3% of U.S. cancer deaths.

Progress in prevention has been made for some occupational factors, such as asbestos, to which workers used to be heavily exposed. Prevention may also become possible for hormone-related cancers such as breast cancer (Henderson *et al.*, 1982), or virus-related cancers such as liver cancer (hepatitis B) and cancer of the cervix (papilloma virus HPV16) (Peto & zur Hausen, 1985).

Further steps in prevention may prove to be difficult. According to Ames *et al.* (1987) we lack the knowledge to do low-dose risk assessment. We also are almost completely ignorant of the carcinogenic potential of the enormous background of natural chemicals in the world.

Both genetic factors and the environment play a crucial role in the origin of cancer. In which part of the multi-stage process leading to cancer these genetic factors are expressed is not known. At least five steps can be distinguished in this process:

1. The causative agent (usually a carcinogenic chemical) enters the body, mostly through surface epithelia.

2. It is converted enzymatically both to inactive products which can be eliminated, and to active carcinogenic forms.
3. The latter can combine with cellular macromolecules and may -in the case of DNA- cause inheritable damage to the cell. This damage can possibly lead to escape of the commitment of cells to senescence leading to a capacity for infinite multiplication preceding malignant transformation.
4. The transformed cell divides to form a tumor.
5. The tumor can subsequently metastasize throughout the body.

1.2 Biotransformation

When consequences of environmental exposure to multiple agents are evaluated with respect to their health effects, metabolism of xenobiotics and metabolic interactions have to be considered.
To facilitate the elimination of xenobiotics the organism uses several enzyme systems that alter the chemical structure of xenobiotics. This generally results in an enhanced water solubility of the products formed. This array of processes, called biotransformation, is indispensable for an organism to eliminate lipophilic compounds.

Biotransformation of xenobiotics may be considered to occur in two phases. Phase I involves reactions like oxidation, reduction, hydrolysis which changes molecules in such a way that they become more hydrophilic and more readily excretable. The most important phase I enzymes are the cytochrome P450 enzymes. Phase II metabolism (conjugation reactions) comprises reactions in which small endogenous molecules (water, glucuronic acid, glutathione, sulphate, glycine and other amino acids) are added to the functional groups of the xenobiotic or its phase I metabolite, making them even more polar.

The so called P450 gene superfamily catalyzes a surprisingly large number of chemical reactions with an almost unlimited number of biologically occurring and xenobiotic compounds (Lu *et al.*, 1980; Black & Coon, 1987; Schwab & Johnson, 1987; Ryan & Levin, 1990; Guengerich, 1991; Porter & Coon, 1991). The P450 gene superfamily encodes numerous enzymes, of which so far more than 150 have been characterized. These vary from about 10 to over 90% in sequence identity and occur in biological sources as diverse as microorganisms, plants, and animals. Almost all mammalian tissues contain one or more of these cytochromes in various organelles, predominantly in the endoplasmic reticulum and mitochondria. Some of the P450 isoforms are fairly specific in their choice of substrates (for example, the steroidogenic cytochromes), but many, and particularly those in the hepatic endoplasmic reticulum, catalyze a large number of reactions with an almost unlimited number of compounds. In the latter category are synthetic environmental chemicals, now estimated at about 250,000, most of

which are potential P450 substrates if not inducers or inhibitors of the individual cytochromes Examples of xenobiotics that serve as P450 substrates are drugs (including antibiotics), antioxidants, organic solvents, anesthetics, dyes, pesticides, alcohols, odorants, and flavorants, and a variety of unusual substances in plants and microorganisms, which, despite their biological occurrence, are foreign to animals The physiologically important substrates include steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids and acetone

The P450 proteins from all sources with 40% or greater sequence identity are included in the same family, as designated by an Arabic number, and those with greater than 55% identity are included in the same subfamily, as designated by a capital letter The individual genes (and gene products) are arbitrarily numbered As an example, the major phenobarbital inducible cytochrome is called P4502B4 or CYP2B4 (enzyme) and the corresponding gene *CYP2B4* (gene)

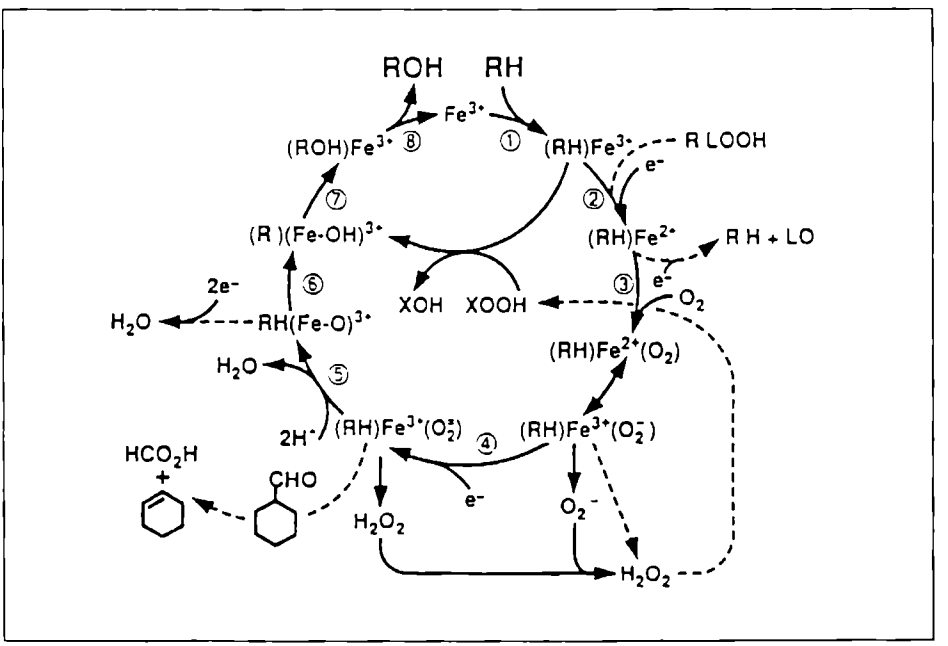


Figure 1 Overall scheme for mechanism of action of P450 Fe represents the heme iron atom in the active site RH a substrate, and ROH the corresponding monooxygenation product R'LOOH represents a lipid hydroperoxide and R'H and LO represent the corresponding reduction products (alkane and oxoacid, respectively) XOOH represents a peroxy compound that serves as an alternate oxygen donor to molecular oxygen

The steps involved in the P450-catalyzed reduction of molecular oxygen with incorporation of one oxygen atom into a substrate, RH, to give the corresponding product, ROH, are shown in Figure 1. (Coon *et al.*, 1992).

After exposure to various compounds increases in total liver P450 content are observed. Generally, this process of induction leads to alterations in the distribution pattern of P450 enzymes and to modification of biotransformation activities. For a number of these enzymes fairly specific metabolic 'marker-substrates' or 'marker-conversions' have been described. However, in control animals or in animals treated with other chemicals, additional P450 isoenzymes may catalyze these reactions as well (Nakajima *et al.*, 1990,1991; Nebert *et al.*, 1991).

Three of the P450 subfamilies are of special interest for the present study; they could play a part in the interaction between ethanol and PAHs. These are the P450 1A, P4502C and P4502E subfamilies.

The enzymes of the P4501A subfamily are induced by 3-Methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) among other (halogenated) aromatic hydrocarbons. Some evidence exists that the P4501A family can also be induced in humans. Many procarcinogens can be activated by these enzymes and P4501A induction is often considered to be associated with increased carcinogenicity (Guengerich, 1988; Ioannides & Parke, 1987,1990), but this is a matter of debate (McKillop & Case, 1991). P450 1A proteins are regulated via interaction of the inducing compound with a cytosolic protein, the *Ah*-receptor (Nebert & Gonzalez, 1987; Nebert & Jones, 1989).

After induction, P4501A1 and P4501A3 can be monitored in rat liver using the ethoxyresorufin O-deethylation (EROD) assay. Quantitatively, the P4501A enzymes are not very important in untreated rats (Sesardic *et al.*, 1990,1990a). In untreated male rats EROD activity is catalyzed to a high extent by other enzymes (P4502C6 and/or P4502C11) (Nakajima *et al.*, 1990, 1991). In benzo(*a*)pyrene-treated C57B1 mice EROD is mainly catalyzed by P4501A2 rather than P4501A1 (Tsyrlov & Duzchak, 1990). In human liver, P4501A2 can be found, induced by smoking.

The largest P450-subfamily comprises the 2C enzymes. Among these are the P450s which are constitutively expressed to high levels. Of the total immunochemically detectable P450 in untreated adult male rat liver, the P4502C11 and P4502C6 enzymes make up for ca 50% and 30%, respectively (Steward *et al.*, 1985). In female rats P4502C11 is hardly expressed but the functionally different P4502C12 form is expressed instead. These and some other forms are regulated by the fluctuation pattern of the plasma growth hormone concentration. (Waxman, 1988; Westin *et al.*, 1990; Zaphiropoulos *et al.*, 1990). P4502C11 can be monitored in rat liver using testosterone (TST) 2α- or 16α-hydroxylation. For P4502C12 determination of 5α-androstene-3α,17ß-diol disulfate 15ß-hydroxylation might be a good indicator activity (Guengerich, 1987;

Waxman, 1988). P4502C8, P4502C9 and P4502C10 enzymes are abundant in human liver and are involved in mephenytoine-4-hydroxylation (Guengerich *et al.*, 1988). In part of the human population a phenotypic deficiency resulting in low mephenytoin metabolism is observed. This deficiency is probably attributable to defective P4502C enzymes (Guengerich, 1987). The human 2C8, 2C9 and 2C10 P450-enzymes are closely related to rat P4502C11 and rabbit P4502C5 (Morel *et al.*, 1990a; Mennes, 1992; Wortelboer, 1992).

An ethanol-inducible form of P450 was purified from rabbits (Koop *et al.*, 1982) and has been characterized from many species, including rats, mice, hamsters, and humans, and it was demonstrated that the purified enzyme will catalyze bioactivation reactions (Lieber, 1984; Garro & Lieber, 1990; Koop & Coon, 1986). Additional observations support the involvement of P4502E1 in ethanol-potentiated hepatotoxicity: other compounds that induce P4502E1, such as acetone and other short-chain alcohols, also potentiate the same hepatotoxicity (Koop & Coon, 1986; Zimmerman, 1986). P4502E1 is located in the cell layers near the terminal hepatic vein and is induced in the same region by all inducers examined (Lieber, 1990). The centrilobular region of the liver is most susceptible to chemical toxins that are substrates for P4502E1. Many compounds have been identified as substrates for P450 2E1, as summarized in Table 1 (Koop, 1992). The substrates are, in general, small, relatively polar compounds. The list includes many solvents used extensively in industry such as benzene, chloroform, and trichloroethylene. As a result, the bioactivation of these types of protoxicants by P4502E1 places particular emphasis on this form of P450 in human health. The enzyme is readily inducible in humans (Umeno *et al.*, 1988; Raucy *et al.*, 1987; Guengerich *et al.*, 1991) and there can be significant exposure to its substrates in the workplace. Chronic ethanol ingestion is not required for induction of P4502E1; significant increases in the enzyme can be observed after a single dose of ethanol. All compounds that have been shown to be substrates in animal models are also substrates for the human ortholog. P4502E1 can be monitored in rat liver using hydroxylation of aniline, *p*-nitrophenol and chlorzoxazone or demethylation of *N,N*-dimethylnitrosamine.

Concerning the possible interactions of ethanol and PAHs, the most interesting enzyme activities of the phase II biotransformation reactions are glutathione conjugation and epoxide hydrolase. Glutathione S-transferases (GSTs) exist in multiple forms which conjugate glutathione on the sulphur atom of cysteine to various electrophiles (Mannervik, 1985; Pickett & Lu, 1989; Coles & Ketterer, 1990; Boyer, 1989). In addition, GSTs bind a variety of hydrophobic compounds such as haem, bilirubin and polycyclic aromatic hydrocarbons (PAHs) with high affinity and have been suggested to act as intracellular transport proteins (Mannervik, 1987). The GSTs are cytosolic proteins, with the exception of a microsomal form identified in rodents. The enzymes are homodimers or heterodimers comprising at least seven subunits. Until now, evidence exists for the

occurrence of more than 11 subunits in the rat and 7 subunits in human (Coles & Ketterer, 1990). GSTs are classified into three different families, named alpha, mu, and pi. This classification is based on catalytic activity, isoelectric points and N-terminal sequences. Class alpha GST consists of rat 1-1, 1-2, 2-2, 8-8 and human α. Class mu consists of rat 3-3, 3-4, 4-4, 6-6 and human μ , class pi consists of rat 7-7 and human π (Mannervik *et al.*, 1987). GSTs are widespread in nature and have been found in almost every rat and human tissue examined. Their expression in different tissues is not uniform, however. In rat liver, subunits 1, 2, 3 and 4 are predominant, whereas in the lung, small intestine and placenta, subunit 7 is the major form. It has been suggested that expression of subunit 7 is a common phenotypic expression of malignant transformation of cells, including malignant liver cells.

To determine the catalytic activity of GSTs the most commonly used substrate is 1-chloro-2,4-dinitrobenzene (CDNB), since it is utilized well by most of the rat and human subunits. Although the products of GST-catalysed reactions are usually non-toxic, the formation of glutathione conjugates of some compounds may cause cell injury. The glutathione conjugate of 1,2-dibromoethane can react directly with DNA forming an adduct that is mutagenic in the Ames assay. Another type of enhanced toxicity is exerted via metabolism of glutathione conjugates of certain halogenated hydrocarbons within the kidney to cysteine conjugates. These may be further metabolized by a renal enzyme (ß-lyase) to a sulphur-containing reactive compound which can initiate injury in proximal renal tubular cells (Coles & Ketterer, 1990).

Epoxide hydrolases (EH) are monomeric proteins important in the degradation of potentially reactive epoxides by the conjugation of water (Oesch, 1980; Timms *et al.*, 1987). Three different forms have been identified in the endoplasmic reticulum and one in the cytosol. The hydrolysis of the reactive and highly toxic ipoxides to dihydrodiols results, in many instances, in a detoxification, but can serve as a precursor of even more reactive dihydrodiol epoxides. For example, from benzo(*a*)pyrene [B(*a*)P] the reactive (+)-*anti*-7,8-diol-9,10-epoxide (Gelboin, 1980) is formed (Section 3 of this Introduction refers to that process). In addition to hydrolysing epoxide metabolites of xenobiotics, epoxide hydrolases catalyse the hydration of endogenous epoxides, such as oestrogen and androgen epoxide, and cholesterol epoxides (Timms *et al.*, 1987).

Substrate	Product measured
Aromatic compounds	
pyridine	pyridine N-oxide
3-hydroxypyridine	2,5-dihydroxypyridine
p-nitrophenol	4-nitrocatechol
benzene	phenol
phenol	hydroquinone, catechol
acetaminophen	glutathione conjugates
pyrazole	4-hydroxypyrazole
chlorzoxazone	6-hydroxychlorzoxazone
styrene	glutathione conjugate
aniline	*p*-aminophenol
Halogenated alkanes and alkenes/alkanes	
chloroform	glutathione conjugate
pentane	product not measured
chloromethane	formaldehyde
dibromoethane	glutathione conjugate
dichloromethane	glutathione conjugates
1,2-dichloropropane	glutathione conjugate
ethyl carbamate	1,N^6-ethenoadenosine
1,1,1-trichloroethane	1,1,1-trichloro-2-hydroxyethane
trichloroethylene	chloral
ethylene dibromide	1,N^6-ethenoadenosine
ethylene dichloride	1,N^6-ethenoadenosine
vinyl chloride	1,N^6-ethenoadenosine
vinyl bromide	1,N^6-ethenoadenosine
vinyl carbamate	1,N^6-ethenoadenosine
enflurane	fluoride
halothane	trifluoroacetic acid
1,1,1,2-tetrafluoro- ethane	fluoride
Alcohols/ketones/nitriles	
ethanol	acetaldehyde
propanol	propionaldehyde
isopropanol	acetone
butanol	butyraldehyde
pentanol	valeraldehyde
glycerol	formaldehyde
acetol	methylglyoxal
acetone	acetol
acetonitrile (+catalase)	cyanide
acrylonitrile	1,N^6-ethenoadenosine
nitrosamines/azocompounds	
N,N-dimethylnitrosamine	formaldehyde/nitrite
azoxymethane	azoxymethanol
methylazoxymethanol	methanol/formic acid
N,N-diethylnitrosamine	acetaldehyde
N-nitrosopyrrolidine	4-hydroxybutyraldehyde
N-nitroso-2,6-dimethylmorpholine	*N*-nitroso-(2-hydroxypropyl)-(2-oxopropyl)amine
Ethers	
diethylether	acetaldehyde
methyl *t*-butyl ether	formaldehyde/*t*-butanol
Reductive substrates	
carbon tetrachloride	lipid peroxidation/ chloroform
chromium (Cr^{VI})	product not measured
13-hydroperoxy-9,11-octadecadienoic acid	pentane
15-hydroperoxy-5,8,11,13-eicosatetraenic acid	pentane
cumyl hydroperoxide	methane/acetophenone
t-butylhydroperoxide	methane/acetone
oxygen	superoxide/peroxide/ water

Table 1. Substrates metabolized by cytochrome P450 2E1

2. ETHANOL

2.1 Human Exposure

The word 'alcohol' stems from Arabic (*al* = the; *kuhl* = 'spirit' or 'refined'). Originally it referred to a fine powder used to darken the area around the eyes, i.e. eyeblack, and is related to the Hebrew *kahol*, a substance outlining the eyes (Caenegem *et al.*, 1980; Keller *et al.*, 1982). In Latin pharmacology the term was used to indicate fine powdery substances or concentrations of distilled liquids. Paracelsus (1493-1541) used the term for 'the best' or 'the spirit' of wine.

The only alcohol suitable for consumption is ethyl alcohol, or ethanol. Ethanol is a clear, colourless liquid, generated by carbohydrate fermentation or by chemical synthesis. In its pure form ethanol may be used for several purposes, for example as a solvent, or -in a 70% solution- as a bactericide. When administered as a medicine, alcohol may have a stimulating as well as a sedative, anaesthetic or narcotic effect. Its effectiveness depends on the concentration in the organism (Majchrowitz, 1985).

Ethanol is mostly consumed voluntarily as part of alcoholic beverages. Used in this way, the effects of alcohol may range from euphoria to intoxication. The predominant types of commercially produced alcoholic beverages are beer, wine (including fortified wine) and spirits (including liqueur-like beverages). In the Netherlands, standard glasses of wine, beer and spirits contain ca. 10g of ethanol each. Glasses of sherry, port and some less current beverages contain a little less alcohol. The ethanol concentration of a beverage is of importance in relation to the damage that can be caused to the gastrointestinal mucosa (Te Wierik, 1991).

The main components of all alcoholic beverages are ethanol and water. Beers also contain substantial amounts of carbohydrates; sweet beverages contain sugar. Besides, alcoholic beverages may contain hundreds of other substances which together characterize the beverage in question. These substances can be classified into macronutrients (carbohydrates and proteins), vitamins, minerals and trace elements, congeners of ethanol, contaminants and additives. Small quantities of carbohydrates, proteins, vitamins and minerals may be present in alcoholic beverages, particularly in wine and beer. They do not contribute significantly to the daily nutritional intake.

Congeners of ethanol can originate from the raw ingredients or be produced during fermentation (methanol, higher alcohols, phenols, acids). Pesticides, asbestos or mycotoxins may be present as contaminants. Additives can be head-retaining agents, flavouring agents and preservatives to prevent the activity of bacteria and moulds. Little is known about the physical effects of these compounds, caused by the mixture of minute quantities of these substances present in alcoholic beverages (Vrij-Standhardt, 1991).

In the seventeenth and eighteenth centuries, spirits shifted from a pharmaceutical status to an item of everyday consumption, as industrial production methods flooded the market. Technological innovations transformed beer production, starting in the latter part of the nineteenth century, from the craft producing beverages for local and immediate consumption to an integrated industry producing a beverage that could be transported worldwide and stored almost indefinitely (Anonymous, 1966). Improvements in agricultural methods and the development of disease-resistant vines have allowed greatly increased wine production and consumption. Thus, wine consumption in France quadrupled in the last decades of the nineteenth century (Johnson, 1985). In general, the advent of industrially produced alcoholic beverages with an indefinite shelf-life, improvements in transportation and development of a cash economy have erased constraints on availability of alcohol. Under these circumstances, constraints on consumption depend on state controls on availability and price and, for example, on religious and social habits (IARC, 1988).

Even in Europe, the current level of availability of alcoholic beverages is a relatively recent historical phenomenon. By the middle of the nineteenth century, intake of alcoholic beverages was high in most countries in Europe and North America, but, at the turn of the century, there was a decline in consumption which continued until the period between the two world wars. Such patterns ('long waves') of consumption of alcoholic beverages are common across very different countries and are not associated with factors commonly proposed to explain these consumption habits, such as buying power, amount of leisure time, social misery or industrialization and urbanisation. The reform movements, including the international temperance movement may be a partial explanation of lower consumption in the first decades of the twentieth century in many industrialized countries (IARC, 1988).

In contrast, the last few decades have been a period of increasing consumption in developed countries, with some countries approaching the peak levels of the nineteenth century. The largest growth rates have been recorded in countries with originally relatively low levels of average consumption. Consumption patterns tend to internationalise. The increase in consumption of alcoholic beverages slowed down or levelled off in many countries in the 1970s, and some decreases were recorded. This phenomenon has been explained partially by economic difficulties experienced in most industrialized countries and by changes in living conditions and life styles (Mäkelä *et al.*, 1981). In the developing countries, the variation in trends over the last 30 years has undoubtedly been greater. Most of the available information points toward increases in consumption, except in areas such as Muslim countries (Moser, 1985).

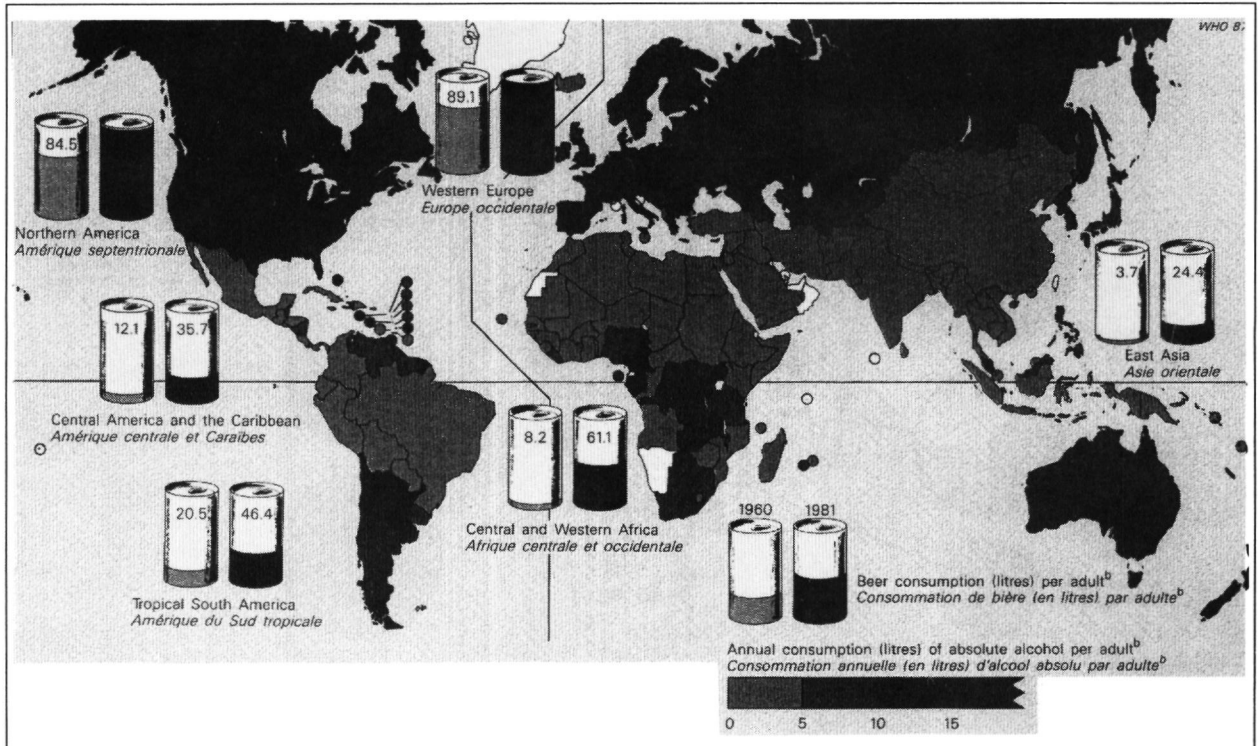


Figure 2. Alcohol consumption around 1977 and trends in beer consumption for selected regions, 1960-1981. Estimates based on data available to WHO (wld hlth statist annu, 1987)

There are wide variations in the rates of consumption of alcoholic beverages between countries and between regions. In Figure 2 the alcohol consumption around 1977 and trends in beer consumption for selected regions 1960-1981 is shown (Wld hlth statist annu, 1987). In 1960, as well as in 1981, commercially produced beer, wine and spirits contributed approximately equal amounts to the world ethanol consumption.

In the Netherlands, the pure alcohol consumption per citizen increased from 2.6 l per year in 1960 to 8.3 l per year in 1988 (de Zwart, 1989). In general, 8 to 10% of the Dutch population of 15 years of age and older has alcohol-related problems (Projectgroep Alcohol Voorlichtings Plan, 1990). There are 200,000 alcoholics and half a million heavy drinkers who consume 8-12 drinks a day (Schoenmaker-Hol, 1988). Although moderate alcohol consumption is hard to define many scientists have tried to do so. McDonald defines two drinks per day for the average woman and three drinks per day for the average man as moderate. Up to 13 drinks per week by women and up to 20 drinks per week by men is regarded as healthy drinking in another study (Anderson, 1986). The Royal College of Physicians suggest 17 drinks per week for men and 11 drinks per week for women to be moderate (Anonymous, 1987).

2.2 Pharmacokinetics

2.2.1 Absorption

Ethanol is absorbed over almost the entire length of the digestive tract. Absorption from the mouth and oesophagus is minimal, some more occurs in the large intestine. A significant proportion of ethanol is absorbed from the stomach (ca. 20%) (Jones & Jones, 1984) and most from the duodenum and small intestine. The ethanol ingested is highly diluted by the admixture of saliva and digestive juices. The ethanol molecules are absorbed through the intestinal wall by a process of passive diffusion (Berggren & Goldberg, 1940); the quantity of ethanol absorbed per unit of time is directly proportional to the concentration gradient between intestinal lumen, epithelial cells, capillaries and the portal vein (Beck & Dinda, 1981). Ethanol in the blood passes almost immediately into brain tissue (Harger *et al.*, 1937). Ethanol absorption follows first-order kinetics.

The presence of food (particularly food rich in fats and carbohydrates) in the stomach delays gastric emptying and consequently postpones the moment the ethanol is absorbed in the intestine. This results in a lower Blood Alcohol Concentration (BAC) peak: the ethanol will be absorbed into the blood over a longer period of time while elimination has already set in. A light meal, taken just before the consumption of whisky or a martini-based cocktail (22-44g ethanol) lowered the BAC peak by 50-80% (Haggard *et al.*, 1941). Ethanol absorbtion may also be delayed by dilution, the presence of congeners, a drop in body

temperature, deep mental concentration or physical exercise Aspirin, aminopyrine and anticholinergic drugs also slow down absorption whereas cholinergic drugs, insulin-induced hypoglycaemis and a raise in body temperature increase the absorption rate slightly (Holford, 1987, Kalant, 1971) Variation in hormonal status (e g stage of the menstrual cycle) affects ethanol absorption (Jones & Jones, 1976) The absorption rate shows a diurnal rhythmicity (Lotterle *et al*, 1989) Ethanol absorption seems also to be under genetic control (Reed *et al*, 1976) In normal circumstances the maximum blood alcohol concentration (BAC) will be reached 45 minutes after the consumption of a single dose (Anonymous, 1972)

2 2 2 Distribution

Absorption of ethanol from the gastrointestinal tract occurs by simple passive diffusion (Wallgren & Barry, 1970) Once ethanol has been absorbed into the bloodstream it is rapidly transported throughout the body and distributed over the body fluids The small, electrically-neutral ethanol molecules can diffuse easily through membranes During absorption the arterial BAC tends to be higher than the venous BAC, peaking at a higher level and requiring less time to reach the peak, until an arterio-venous concentration equilibrium is reached, whereafter the venous BAC remains above the arterial BAC (Martin *et al*, 1984) About 1-1½ h after drinking an equilibrium is reached (Pawan, 1972) This will take place sooner in organs highly perfused with blood (brain, lungs, kidneys, liver) than in other organs (Wallgren, 1970) Once the equilibrium has been reached the amount of ethanol in the organs will be proportional to the organs' water content Body fat and skeletal mass hardly absorb any ethanol Therefore, an identical quantity of ethanol per kg body weight will induce a higher BAC in women than in men Because women usually also have a lower body weight than men, the differences in BAC between the 'reference woman' and the 'reference man' will be even greater after ingestion of an identical amount of ethanol The amount of body water of the reference women (58 kg) is only 69% of that of the reference man (70 kg) Besides, a difference in 'first-pass metabolism' of ethanol in the stomach of males and females was found recently, man having a larger amount of ADH present (Frezza *et al*, 1990)

2 2 3 Elimination

During elimination, when the arterio-venous concentration equilibrium is reached, there is a close relationship between ethanol concentrations in breath, arterial blood and venous blood (Martin *et al*, 1984) so the blood alcohol concentration can be calculated from the breath value

Of the ethanol absorbed 90-98% is oxidized to CO_2 and H_2O, 1-5% is excreted in an unaltered state in urine, breath, sweat and tears and another 1-5% is expired via the lungs.

At all times the excretion of ethanol is a function of the actual BAC, so the total amount of excreted ethanol can be seen as a function of the area under the BAC-time curve (AUC). There is a non-linear relationship between oral ethanol dose and AUC (Wilkinson, 1980). An oral dose of 40 g alcohol for a person of 70 kg results in an AUC of about 3 g.h/l. Assuming a breath/blood partition coefficient of 1:2100 and a lung ventilation rate (in rest) of 8 l/min, 720 mg of ethanol -2% of the dose- is expired. Alcohol is not concentrated in urine (Olson, 1979). The blood/urine ethanol concentration ratio is around 1 (0.5-1.0 in the absorption phase, 1.2-2.0 in the elimination phase) (Schmidt, 1987). With an average urine production of 1.5 l/24h, 0.5% of a dose of 40 g ethanol is estimated to be excreted via the kidneys.
The amount of ethanol excreted in sweat is negligible except in case of excessive perspiration, where it can reach 0.5% (Mallach, 1987).

The height of a Blood Alcohol Curve (BAC) at any given moment will depend on the amount of ethanol absorbed at that particular moment, its distribution over the body, and the amount of ethanol already converted and excreted (Wilkinson, 1980). The BAC curve first shows a steep increase (absorption phase), followed by a slow decrease (elimination phase). Holford (1987) found a linear relationship between oral ethanol dose and BAC peak; C_{peak}(mg/l) = 20*dose (g/70kg).
Two functions can be used to describe the elimination of ethanol from the blood: a straight line (zero-order kinetics according to Widmark), and the Michaelis-Menten equation (Lundquist & Wolthers, 1958; Wagner, 1976).

Widmark equation: $C = C_0 - \beta t$
C concentration at a given moment t
C_0 initial concentration
β slope

Michaelis-Menten equation: $C_0 - C + K_M \ln C_0/C = V_m t$
K_M Michaelis-Menten constant
V_m maximum velocity
others symbols are the same as the Widmark equation

The Michaelis-Menten equation expresses more adequately the experimentally obtained course of the curve than Widmark's straight line approach does. Particularly the tail of the curve fits the Michaelis-Menten equation. Yet it remains feasible to approach the elimination of ethanol from the blood by means of a

straight line, depending on the specific application required. The zero-order kinetics approach is simple, but less precise since it can be applied only to BACs exceeding 0.2 g/l. When applied to oral consumption of ethanol, absorption and distribution often being disturbing factors, this method usually is sufficiently accurate. The Michaelis-Menten equation covers the whole of the elimination phase, even when concentrations are low. Its application, however, requires more complicated calculations.

The mean elimination rate from blood is 100 mg/kg bodyweight per hour with a range of 88-132 mg/kg per hour. Depending on individual elimination rate and body weight, an amount varying between 4.5 g and 12 g ethanol may be processed per hour.

2.2.4 Metabolism

The rate of ethanol metabolism varies among individuals, and studies of twins indicate that interindividual variability in the rate of ethanol metabolism is under genetic control (Vesell *et al.*, 1971; Kopun & Propping, 1977).

Ethanol is eliminated from the body mainly by metabolism in the liver and only minimally by urinary excretion and pulmonary exhalation (Wallgren & Barry, 1970). Other tissues such as kidney (Lelair & Muñoz, 1938), and intestine oxidize ethanol to a small extent only. This was also thought of metabolism in the stomach, until it was recently discovered that indeed the stomach can attribute considerably to ethanol metabolism. This metabolizing capacity is much lower in women compared to men, and in alcoholics compared to normal drinkers.

The hepatic metabolism of ethanol proceeds in three basic steps. First, ethanol is oxidized by alcohol dehydrogenase (ADH) within the cytosol of hepatocytes to acetaldehyde; second, acetaldehyde is converted by aldehyde dehydrogenases (AlDH) to acetate, mainly in the mitochondria; and third, acetate produced in the liver is released into the blood and is oxidized by peripheral tissues to carbon dioxide, fatty acids and water (citric acid cycle). The main pathway for ethanol metabolism proceeds via alcohol dehydrogenase (ADH). However, alternative pathways for ethanol oxidation have been described, which are situated in other subcellular compartments.

ADH occurs in the soluble fraction of the liver and is also found in other tissues in the body, such as gastrointestinal mucosa, kidney, lung and possible brain (Mistilis & Garske, 1969; Hawkins & Kalant, 1972). It catalyses the oxidation of alcohols, including ethanol, to their corresponding aldehydes in the presence of NAD, according to the following scheme:

$$RCH_2OH + NAD^+ \longrightarrow RCHO + NADH + H^+$$

(Hawkins & Kalant, 1972). The rate of elimination of ethanol *in vivo* correlates with the basal metabolic rate, indicating that the rate of mitochondrial NADH

oxidation is a major rate-limiting step in the ADH pathway. Although increased ADH activity is not associated with increased rates of ethanol oxidation, reduction in ADH activity does lead to a decrease in ethanol elimination (Lieber, 1983).

In 1965, a NADPH-dependent ethanol oxidase was found in microsomes from pig's liver which catalyses the oxidation of methanol to formaldehyde and that of ethanol to acetaldehyde (Orme-Johnson & Ziegler, 1965). This system, which was subsequently designated the microsomal ethanol-oxidizing system (MEOS; Lieber & DeCarli, 1968), carries out the following reaction:

$CH_3CH_2OH + NADPH + O_2 + H^+ \longrightarrow CH_3CHO + NADP^+ + 2H_2O$ (Lieber, 1970)

Human ADH is coded by three structural gene loci, the corresponding products α–, ß- and γ-polypeptides combine to form active dimeric isozymes. The ADH molecule may appear in at least nine electrophoretically different isozyme forms (Smith *et al,* 1971, 1973). An 'atypical' ADH has been described which differs from the usual enzyme in its catalytic activity, pH optimum, kinetic parameters and molecular structure (Yoshida *et al,* 1981). In European countries the incidence of atypical ADH ranges from 4 to 20% (von Wartburg & Schürch, 1968). In Japan, however, 85-98% of the population carries the atypical ADH (Agarwal & Goedde, 1986). In spite of the presence of highly active atypical ADH, however, the rate of ethanol metabolism in normal and atypical ADH phenotype carriers is not significantly different. Other isozyme forms found in human liver include π-ADH (Li *et al.,* 1977) and ADH-Indianapolis (Bosron *et al.,* 1980).

Ethanol binds to hepatic cytochrome P450 and gives a modified type-II binding spectrum (Rubin *et al.,* 1971). The K_m of cytochrome P450 for ethanol (about 8 mM) is greater than that of ADH (Lieber & DeCarli, 1970; Lindros *et al.,* 1974). An isozyme of cytochrome P450 that is induced by ethanol, IIE1 (previously described as $P450_{3a}$, $P450_{ALC}$or P450j; Nebert *et al.,* 1987) has been purified from the liver of rabbits (Koop *et al.,* 1982), rats (Peng *et al.,* 1982), hamsters, deermice and baboons (Lasker *et al.,* 1986a). Its presence has been demonstrated in rabbit kidney and nasal mucosa (Ding *et al.,* 1986; Ueng *et al.,* 1987) and in rat kidney (Thomas *et al.,* 1987), but not in microsomes from a variety of other tissues (Ding *et al.,* 1986). Cytochrome P450-dependent ethanol oxidizing capacity has also been demonstrated in mucosal cells from the upper gastrointestinal tract and colon of rats (Seitz *et al.,* 1979, 1982) and in macrophages from a variety of tissues in mice (Wickramasinghe *et al.,* 1987).

Catalase is a haemoprotein located in the peroxisomes of many tissues (de Duve & Baudhuin, 1966). An early suggestion that catalase might play a role in the metabolism of ethanol (Keilin & Hartree, 1936) was later confirmed by Laser (1955), who showed that ethanol could be oxidized effectively in the

presence of hydrogen peroxide and catalase. Catalase can oxidize ethanol *in vitro* only in the presence of a hydrogen peroxide generating system.

During the second step in the oxidation process the acetaldehyde generated is converted into acetate by aldehyde dehydrogenase (AlDH). A NAD-dependent AlDH with a broad substrate specificty for aldehydes was described in 1949 (Racker). The enzyme has a very low K_m value and a high reaction rate (Grunnet, 1973), therefore, normally, only low concentrations of acetaldehyde are found outside the liver (Jacobsen, 1952; Kiessling, 1962; Review; IARC, 1985). At low concentrations of acetaldehyde (<50 M) during ethanol oxidation, acetaldehyde oxidation is predominantly a mitochondrial process (Grunnet, 1973; Marjanen, 1973; Lindros *et al.*, 1974; Parrilla *et al.*, 1974). Several isozymes of AlDH have been identified (reviews Lindros, 1978; Salaspuro & Lindros, 1985). Human AlDH is a tetrameric enzyme with a molecular weight of ca. 2,300,000. In the liver at least four different isozymes exist, which differ in structural and functional properties such as primary structure, molecular weight, electrophoretic migration, isoelectric point, catalytic constants and subcellular distribution. $AlDH_2$ has the highest affinity to alcohol. $AlDH_2$ is located in the mitochondria, the other AlDH isozymes in the cytosol. Cytosolic AlDH is more susceptible to inhibition by disulfiram (Alderman *et al.*, 1982).

Whites have two predominant AlDH isozymes: $AlDH_1$ and $AlDH_2$. Many Asians do not have the active $AlDH_2$ isozyme, but an inactive form (Goedde & Agarwas, 1987). This variant is found in about 50% of Japanese, 35% of Chinese and 40% of Vietnamese and Indonesians. The lack of the active form of this AlDH isozyme, causing an increased acetaldehyde level upon alcohol consumption, is considered the main cause of the flushing syndrome (Harada *et al.*, 1981; Yoshida, 1983). This is a hypersensitivity to alcohol frequently seen in people belonging to the mongoloid race, manifesting itself in cardiac arrhytmia and flushing. These phenomena are comparable with the disulfiram reaction. They are considered to result from acute acetaldehyde intoxication. The atypical ADH isozyme demonstrable in ca. 90% of all orientals is now considered of little importance as a cause of flushing (Lindros, 1982; Ricciardi, 1983). Alcohol ingestion by Orientals resulted in marked elevations of blood acetaldehyde levels, with concentrations ranging from 0.4 to 3 mg/l (Ijiri, 1974); and individuals developed facial flushing and tachycardia as a direct consequence of elevated blood acetaldehyde levels. Tissues other than the liver may also produce acetaldehyde after ethanol administration (Baraona *et al.*, 1985), and intestinal bacteria have been shown to produce small amounts (Baraona *et al.*, 1986).

It is not clear whether, in the liver, free acetate or acetyl-CoA is generated from ethanol (Wallgren & Berry, 1970). The acetate (or acetyl-CoA) formed in the liver is hardly oxidized during the citric acid cycle because of an NAD^+ shortage in the liver caused by previous oxidation of ethanol and

acetaldehyde (von Wartburg, 1976). Most of the acetate is released into the circulation and oxidized extrahepatically to CO_2 and H_2O through acetyl-CoA in the citric acid cycle.

Extrahepatic dissimilation of acetate yields about two thirds of the total combustion energy produced by ethanol. The remaining energy is released during the generation of acetate in the liver. If completely converted via the ADH pathway, ethanol yields 30 kJ/g energy. In case of habituation to large quantifties of ethanol, it yields less energy owing to a different metabolic mechanisms. In comparison, the energy yield of both carbohydrates and proteins is 17 kJ/g, and of fats 38 kJ/g. Alcoholic beverages may be considered as food-stuffs which are neither useful nor indispensable to the anabolism of the human body, however.

2.3 Health Effects

Alcohol consumption is associated with many health problems, which can be divided into three main types: chronic physical problems, casualty and disability problems, and mental problems.

Physical health problems include malfunction and cirrhosis of the liver, cancers at various sites, effects on the developing embryo and fetus, and other diseases affecting the gastrointestinal, cardiovascular, respiratory, nervous, immuno and reproductive systems (World Health Organization, 1980). Of all patients in a regular hospital, 25-40% are dependent on alcohol. However, in general, physical health problems only occur if the alcohol consumption is relatively high. Among moderate drinkers (maximal 2-3 drinks a day) a lower total mortality has often been found in relation to alcohol intake (Shaper *et al.*, 1988). This is due to the reduced incidence of coronary heart disease in this group.
Acute symptons associated with diverse alcohol promillages are given in Table 2.

2.3.1 Effects of ethanol on the liver

The organ most frequently affected by excessive ethanol consumption is the liver. Alcoholic liver disease ranges from fatty liver, alcoholic hepatitis and fibrosis to irreversible cirrhosis. Fatty liver, also called steatosis, is a reversible disorder, in which fat accumulates in the liver cells. In the case of hepatitis much of the liver tissue is destroyed (necrosis) causing subsequent inflammation. Alcoholic cirrhosis is characterized by hepatic fibrosis. The normal structure of the liver and liver functions are irreversibly disturbed.

Chronic alcohol abuse results in functional and structural changes in liver cell mitochondria (Lieber, 1973). This in turn leads to a decrease in fatty acid oxidation and thus promotes accumulation of fat. A decreased production or decreased release of lipoproteins also adds to fat accumulation (Koga &

Hirayama, 1968; Lieber, 1975). In Figure 3, possible mechanisms of fatty liver production through either increase (-->) or decrease (-ll->) of lipid transport and metabolism are shown (Lieber, 1990).

Promillage mg/l	clinical features
500-1500	mild intoxication emotional lability, slight impairment of visual acuity, muscular coordination and reaction time
1500-3000	moderate intoxication visual impairment, sensory loss, muscular incoordination, slowed reaction time and slurred speech
3000-5000	severe intoxication muscular incoordination, blurred or double vision, sometimes stupor and hypothermia, occasionally hypoglycaemia and convulsions
>5000	coma depressed reflexes, respiratory depression, hypotension and hypothermia Death may occur from respiratory or circulatory failure or as the result of aspiration of stomach contents in the absence of the gag reflex

Table 2 Clinical features of ethanol intoxication (Weatherall *et al*, 1983)

Hepatitis and cirrhosis seem to have multiple causes:
-hypoxia (French, 1989; Gavaler, 1982; Israel, 1979)
-increased peroxidation (Aaseth *et al.*, 1986; Dicker & Cederbaum, 1988; Ingelman-Sundberg *et al.*, 1987)
-abnormal cellular immunological activity and disturbed lymphocyte activity (Anonymous, 1983; Barry, 1988; Eddleston & Vento, 1988)
-changes in collagen production (Gavaler, 1982; Horrobin, 1980,1987)
-mitochondrial injury caused by ethanol itself or by acetaldehyde (Lieber, 1980)
-interference with essential fatty acid and prostaglandin metabolism (Glen *et al.*, 1987)
-an antitubular effect of alcohol or acetaldehyde, causing the formation of Mallory bodies (Isselbacher, 1977).

As opposed to some thirty years ago, nutritional deficiencies are now not regarded as the primary cause. Alcohol consumption itself, rather than the attendant malnutrition in alcoholics, plays a key aetiological role in the development of liver injury (Achord, 1988; Lieber, 1982).

Hypoxia seems to be an important factor in hepatic damage, because necrosis and later fibrosis will first be observed in the perivenular zones of the cells. Israel *et al.* (1979a, 1979b) consider necrosis in these zones as caused by a disturbance of the equilibrium between the availability of oxygen and the liver's oxygen requirement. It has been demonstrated that liver cells have an increased oxygen demand during ethanol metabolism, but this can be fully offset by a parallel increase in bloodflow. According to French (1989) chronic ethanol ingestion leads to hepatocellular injury only if multiple factors are operative in combination to induce centrilobular hypoxia. Possible factors are a shift in redox

state, MEOS (Microsomal Ethanol Oxidizing System) induction, a high blood alcohol concentration (BAC), a diet rich in polyunsaturated fatty acids (PUFA), and episodic decreased oxygen supply to the liver.

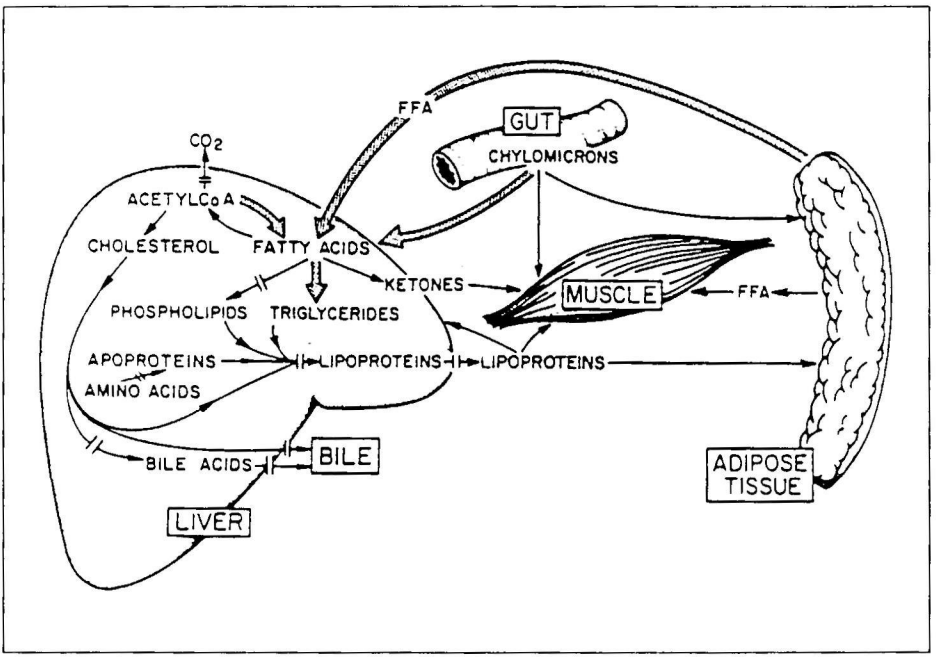


Figure 3. Possible mechanisms of fatty liver production through either increase (-->) or decrease (-II->) of lipid transport and metabolism (from Lieber, 1982)

In 1957 the first report was published about a NADPH-dependent production of hydrogen peroxide in liver microsomes (Gillette *et al.*, 1957). Twenty years later it was shown that a catalytically active rabit liver microsomal P450 system produces hydrogen peroxide. In the presence of substrate, as much as 55% of the consumed oxygen can result in hydrogen peroxide formation, whereas in the absence of substrate, up to 100% of oxygen can be transformed to hydrogen peroxide (Nordblom & Coon, 1977). Nordblom & Coon also found that the nature of the substrate alters the coupling efficiency of the enzyme. The rate of hydrogen peroxide production in isolated perfused rat liver has been determined by spectral intermediates of catalase and the value obtained was 82 nmol H_2O_2/min per g liver. This would give an estimated steady state concentration of 10^{-9}-10^{-7}M hydrogen peroxide in the liver (Oshino *et al.*, 1973). Premereur *et al.* (1986) have found evidence for the existence of a basal P450-dependent hydrogen peroxide

production *in vivo* in guinea pig. Hydrogen peroxide can be produced by dismutation of superoxide anions.

$$2\,O_2^{\cdot-} + 2\,H^+ \longrightarrow 2\,H_2O_2 + O_2$$

This reaction is catalyzed by superoxide dismutase but can also occur spontaneously at a slower rate.

The oxycytochrome P450 complex can be autooxidized by loss of the superoxide anion (Figure 1, par 1.2) and the microsomal hydrogen peroxide production appears to result from dismutation of the liberated superoxide anions. In a non-heme iron catalyzed Haber-Weiss reaction hydroxyl radical formation in biochemical systems could be realized.

Free radicals are believed to play a role in the pathogenesis of ethanol-induced liver damage. In figure 4 reactions of reactive free radicals are given.

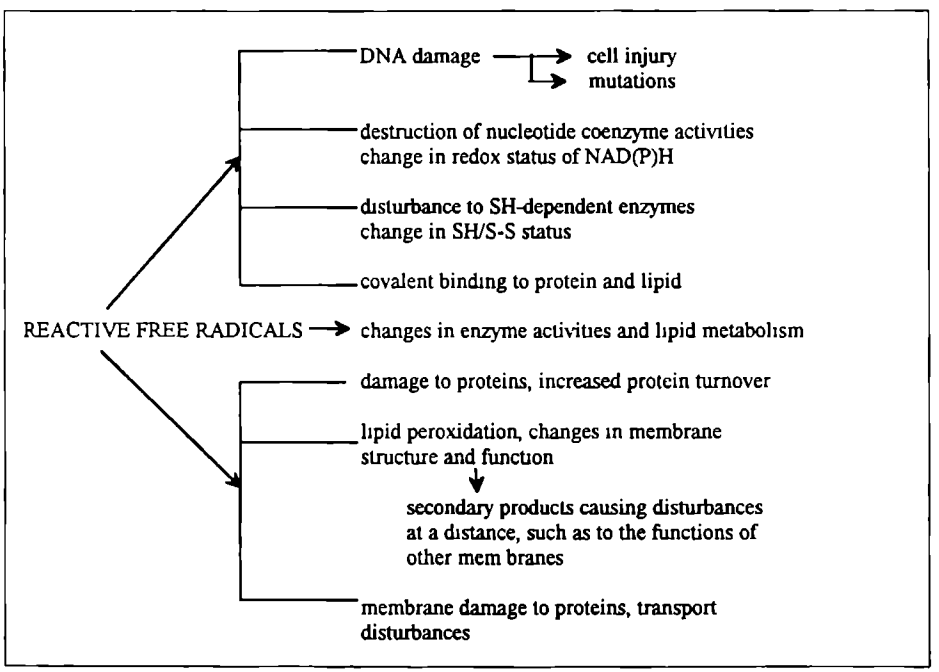


Figure 4. Reactions of reactive free radicals (from Slater, 1987)

The vulnerability of a tissue to peroxidation is a function of the balance between pro-oxidant and antioxidant systems. Glutathione (GSH), ascorbic acid, selenium

and α-tocopherol have antioxidant properties. Ethanol seems to influence both systems at various sites. Recently, the in vivo formation of a free radical metabolite of ethanol (α-hydroxyethyl radical) has been demonstrated (Knecht *et al.*, 1990). Johansson *et al.* (1988) suggest that an enzymatic Haber-Weiss reaction in the active site of the ethanol inducible P450 enzyme. Proposed schemes for possible mechanisms of P450 dependent ethanol oxidation are presented in figure 5.

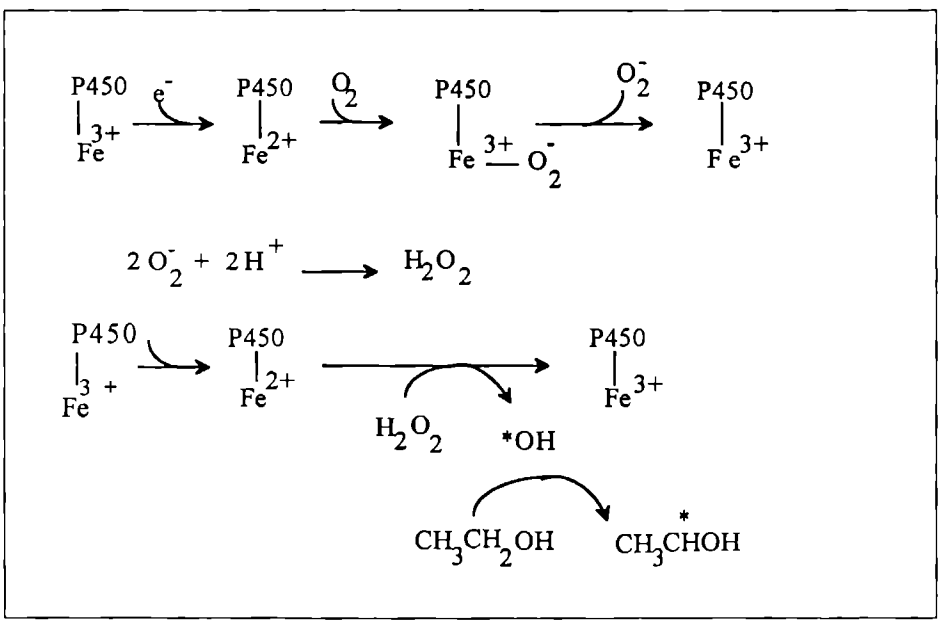


Figure 5. Proposed mechanism for ethanol oxidation catalyzed by the etanol-inducible cytochrome P450. From Johansson (1988).

Acetaldehyde is also a substrate of free radical production (Reinke *et al.*, 1988). In this way, both acute ethanol consumption (direct and via acetaldehyde) and chronic ethanol consumption (by inducing MEOS) promote free radical production, which may contribute to the hepatotoxic effects of ethanol.

2.3.2 Cancer Epidemiology

In France, in 1910, it was observed that about 80% of patients with cancer of the oesophagus and cardiac region of the stomach were alcoholics, who drank mainly

absinthe (Lamy, 1910). In the first half of the century it was noted from mortality statistics in various countries that high risks for cancers of the oral cavity, pharynx, oesophagus and larynx occurred among persons employed in the production and distribution of alcoholic beverages (Young & Russell, 1926; Clemmesen, 1941; Kennaway & Kennaway, 1947; Versluys, 1949). Cancers at these sites occur at lower rates in people who take no alcohol, such as Seventh-day Adventists (Wynder *et al.*, 1959; Lemon *et al.*, 1964; Phillips *et al.*, 1980). Subsequent to historical observations and studies of religious groups, analytical studies of the cohort and case-control type have been carried out.

In descriptive studies, a very crude level of alcohol intake is typically inferred for a group of individuals, on the basis of characteristics such as treatment for alcoholism. Frequently, even measurements of average alcohol intake in these groups and in the groups with which they are compared are lacking.

In case-control and cohort studies involving individual subjects, measurements of alcohol intake are usually obtained by structured interviews or questionnaires. The questions asked vary widely among studies, providing markedly different levels of details about alcohol intake (Room, 1979). In some studies, a single question was asked that provided only a few categories of alcohol consumption. In many studies, separate questions were asked regarding the average frequency (usually in terms of standard units) of drinking beer, wine, spirits and other specific beverages. This information allows a calculation of usual total ethanol intake as well as an estimation of that from the specific beverages. In general, details about intraindividual variations, such as 'binge drinking', have not been incorporated in studies. The validity of self-reported alcohol consumption has been reviewed by Midanik (1982). In some populations (Pernanen, 1974), self reporting of alcohol intake results in a lower total than that for alcohol sales. However, even if a population as a whole tends to underestimate its intake, this may not necessarily be true of participants in epidemiological studies, such as those who volunteer to enrol in a cohort study. Moreover, there is some evidence that underestimation tends to be proportional to consumption, so that at least the broad ordering of respondents is correct (Boland, 1973).

Descriptive studies of the relationship between alcohol consumption and cancer risk entail analysis of the covariation of population-based measures of those two variables. Variations (known or inferred) in alcohol consumption by time, geographic location and category of person are examined in relation to variations in cancer incidence or mortality rates. Since alcohol consumption tends to be associated with other forms of behaviour that might also influence the risk of developing cancer (especially cigarette smoking and aspects of diet), and for which equivalent measures of exposure are frequently not available, it is not possible in descriptive studies to infer a causal relationship between alcohol consumption and cancer risk. Descriptive studies have been used most frequently to study alcohol consumption in relation to specific cancers of the upper

alimentary tract and larynx. Oesophageal cancers, in particular, have been studied in this way in both developed and developing countries. Many geographic correlation studies have been carried out to examine mortality from alimentary tract cancer in relation to mortality from liver cirrhosis and alcoholism within the departments of France (Lasserre, 1967). These studies have consistently shown a strong correlation of oesophageal cancer with the index of alcohol consumption; less strong correlations have been seen for cancers of the mouth, pharynx and stomach.

The relationship between alcohol intake and cancer at a variety of sites has been assessed in many large cohort studies. With few exceptions, detailed information on type of beverage, amount drunk and on smoking was not available. Tobacco smoking and alcohol drinking are often correlated at the individual level, and tobacco smoke is a cause of cancer at many sites that may also be related to alcohol consumption (IARC, 1986). However, a major methodological advantage of cohort studies over case-control studies is the lesser probability of selection bias and bias with regard to information on exposure. The most detailed evidence about the relationship between alcohol and cancer at individual sites has come from case-control studies.

From the human carcinogenicity data it can be concluded that the occurrence of malignant tumours of the oral cavity, pharynx, larynx, oesophagus and liver is causally related to the consumption of alcoholic beverages. Overall, studies on cancers of the urinary bladder, kidney, ovary, prostate and lymphatic and haematopoietic system show no association with consumption of alcoholic beverages. The sparsity of the observations on cancers of the skin, corpus and cervix uteri, vulva, testis, brain, thyroid and soft tissues precludes an evaluation (IARC, 1988).

2.3.3 Ethanol Related Mechanism of Carcinogenesis

It is not clear how the influence of alcoholic beverages on the pathogenesis of cancer of the mouth, pharynx, larynx, oesophagus and liver can be explained; the beverages may be carcinogenic in themselves, or be active as a cocarcinogen; either ethanol or other additives may cause those effects. Indirect effects of alcohol use may also play a part. There is no evidence from animal studies that ethanol *per se* is carcinogenic. However, ethanol may increase the susceptibility of various tissues to chemical carcinogens by a variety of mechanisms (Swann, 1984). Among these are alteration of the metabolism and/or distribution of carcinogens, interfering with the repair of carcinogen-mediated DNA alkylation and the immune response, and stimulation of cellular regeneration. Regarding carcinogenesis in sites that are directly exposed to alcoholic beverages, a local effect has been suggested. Figure 6 shows the possible sites for application in carcinogenesis.

Ethanol itself is unlikely to be carcinogenic. Experiments performed to induce cancer with ethanol in well-fed laboratory animals without liver injury have failed so far (Keller, 1980). Fusel alcohols, i.e. the higher alcohols present in all alcoholic beverages in varying quantities, contribute to the specific taste and flavour of these beverages. Several animal experiments have shown that fusel alcohols (in far greater quantities than usual) are carcinogenic (Gibel, 1975; Obe, 1979). Small quantities of nitrosamines, known to be carcinogenic, have been found in African maize beer, German beer and distilled maize beverages (McGlashan, 1969) while in the areas where these beverages are consumed, the incidence of cancer of the oesophagus is high (Cook, 1971). In cider distillates, small quantities of nitrosamines have also been found.

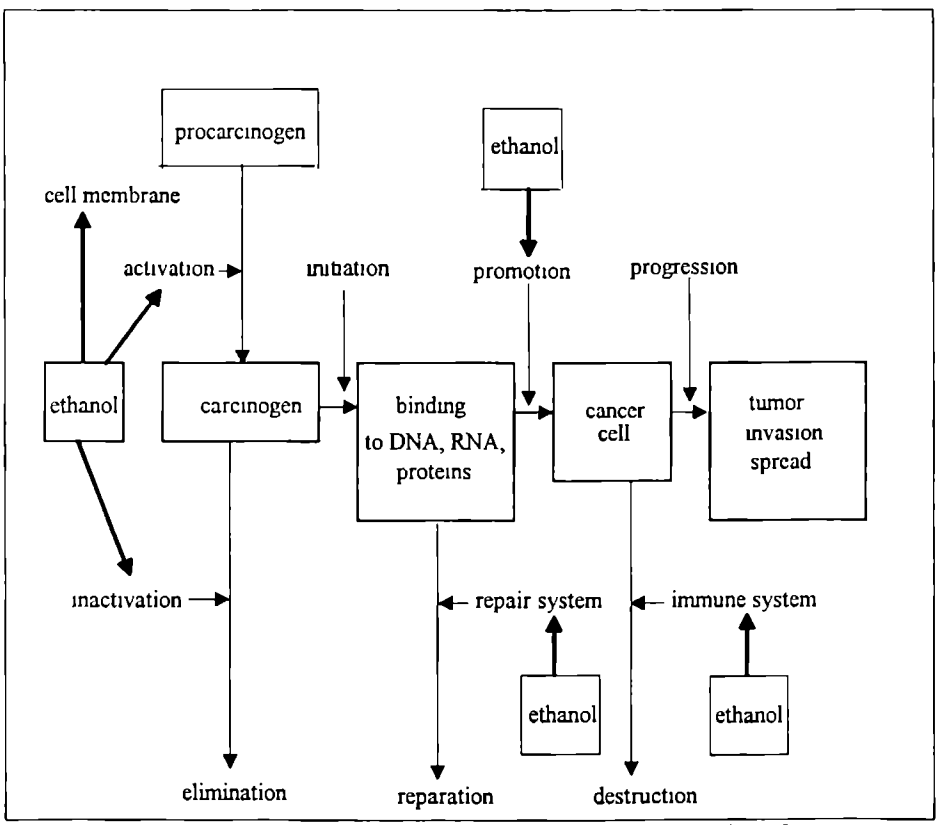


Figure 6. Scheme of two-step carcinogenesis and possible sites of action of ethanol (after Seitz, 1988)

Some suggest this as a possible explanation of the fact that mortality from cancer of the oesophagus in France is highest in Normandy and Brittany, where

consumption of this kind of alcoholic beverages is highest. Whisky and some types of beer (especially the dark types), contain traces of nitrosamines (Keller, 1980) which are formed when the malt is roasted. Furthermore, the presence of ethanol seems to catalyse the production of nitrosamines from their constituent compounds, namely nitrites and secondary amines, under the conditions encountered in the upper gastrointestinal tract (Pignatelli *et al.*, 1976). Alcoholic beverages are extremely complex mixtures in which hundreds of substances may be present. One or more of these can contribute to carcinogenesis. Mutagenesis investigations have revealed mutagenic activity in several commercially produced and home-made apple brandies as well as in red wine and in red grape juice. A variety of carcinogens such as polycyclic hydrocarbons (Matsuda *et al.*, 1966) and asbestos fibres (Wehman, 1974) have been detected in alcoholic beverages.

Acetaldehyde, the major intermediary metabolite of ethanol, was tested for carcinogenicity in rats by inhalation and in hamsters by inhalation and by intratracheal instillation. It produced tumours of the respiratory tract following inhalation, particularly adenocarcinomas and squamous-cell carcinomas of the nasal mucosa in rats and laryngeal carcinomas in hamsters. In hamsters, it did not result in an increased incidence of tumours following intratracheal instillation. Inhalation of acetaldehyde enhanced the incidence of respiratory-tract tumours induced by intratracheal instillation of benzo(*a*)pyrene in hamsters (IARC, 1987).

Nutritional deficiencies are very common in alcoholics. A poor nutritional status seems to be a risk factor for oesophageal cancer (Ziegler, 1981). Chronic alcohol consumption has striking effects on tissue vitamin A levels. Considering the enhancing effects of vitamin A deficiency on carcinogenesis, it is conceivable that part of the interaction between alcohol and cancer may be caused at least indirectly by the alteration of the vitamin A status induced by chronic alcohol abuse (Leo *et al.*, 1986). Deficiencies of some other vitamins (riboflavin, vitamin B6, vitamin E) have also been implicated in the pathogenesis of cancer (Lieber *et al.*, 1986).

Hepatitis B virus (HBV) is associated with an increased risk of hepatocellular carcinoma (Liaw *et al.*, 1986), and alcoholics have an increased risk of HBV infection (Mills *et al.*, 1979). An association between cirrhosis and hepatocellular carcinoma has also been observed (Johnson *et al.*, 1978).

Chronic ethanol consumption can lead to a reduced salivary secretion rate (Dutta, 1989). Since saliva seems to be able to protect the mucosa against the local actions of chemical carcinogens, reduction of the salivary gland function might play a role in carcinogenesis of the upper digestive tract.

Ethanol causes changes in the permeability of membranes (McGoy & Wynder, 1979; Obe & Ristow, 1979) which may increase the diffusion of carcinogenic substances from tobacco smoke and other sources into the cells.By acting as a solvent for carcinogens ethanol may increase its activity.

As a another possible mechanism MEOS induction has been suggested. Regular alcohol consumption induces the microsomal ethanol-oxidizing system (MEOS) in the liver and in the intestine which is associated with an increase in various constituents of the microsomal system, namely phospholipids, cytochrome P450 reductase and cytochrome P450. The bioactivation of procarcinogens into their reactive intermediates in the intestine and/or liver can also be stimulated via this induction (Lieber *et al.*, 1979; Obe & Ristow, 1979; Seitz *et al.*, 1978).

There are two effects of ethanol on DNA that might be associated with cocarcinogenic activity, namely its effect on sister chromatid exchanges (SCEs) and on DNA repair. Obe and Ristow (1979) found an elevation of chromosomal aberrations in the lymphocytes of alcoholics. Results of animal studies indicate that chronic alcohol consumption inhibits the activity of the DNA repair enzyme O^6-methylguanine transferase. However, it remains to be determined whether ethanol also inhibits human O^6-methylguanine transferase (Garro *et al.*, 1986).

DNA demethylation might be related to tumour development by enhancing gene expression or inhibiting chromatin condensation. Inoue *et al.* (1988) noted that demethylation of exon 2 of the c-myc gene of the hepatocellular carcinoma is more (albeit not significantly more) frequently encountered in groups of heavy drinkers.

Several studies have shown an association between heavy drinking and decreased immune response (Mufti *et al.*, 1989). Ethanol-associated immunosuppression has been considered a possible factor contributing to an increased risk of cancer (Daynes *et al.*, 1979). For the most part, however, studies in which decreased immune response has been associated with alcohol abuse involved patients who already had alcoholic liver disease. Therefore, it is difficult to assess whether the immunological defects observed were due directly to alcohol or reflected other aspects of the ongoing disease process. There is reason to question the significance of immunosuppression in general carcinogenesis. Although cancer incidence is higher among immunosuppressed patients and animals, the cancers observed are mostly cancers of the immune system itself (Baird *et al.*, 1982). Nevertheless, the immune system may play a vital role in the defence against virally induced tumours, particularly in HBV-associated hepatocellular carcinoma (Garro & Lieber, 1990).

2.4 Exposure Models for Rats

Historically, the primary reason for doing research on alcohol is that it causes a multitude of problems in humans. The research was mainly concerned with alcoholism and its pathological consequences. The goal of the research was the development of a better understanding of the mechanisms and underlying causes of these problems and ultimately the discovery of ways to prevent or cure the

problems (Eriksson, 1980). The first animal model on alcohol research was used by Richter in 1926, who caried out studies on rats' voluntary choice between an alcohol solution and water.

Much of the research of relevance for humans cannot be conducted on human beings for ethical or technical reasons. Especially for ethanol, it is of importance that using an animal model, it is theoretically possible to examine the effects of alcohol *per se*, without interference from such confounding variables as poor nutritional status, multidrug abuse, prior exposure to alcohol or other drugs, and organ damage. Animal models have been developed in many areas of alcohol research. Selective rodent strains have been bred with either low or high preference for alcohol (Eriksson, 1968; Elmer *et al.*, 1988) in order to identify biochemical, physiological and behavioural differences between those strains. The strains best studied are the AA and ANA rats bred at the research laboratories of ALKO in Finland and the P- and NP-rats from the laboratory in Indianapolis, USA. AA- and P-rats consume large and ANA- and NP-rats very small amounts of alcohol (Lumeng *et al.*, 1982; Waller *et al.*, 1984). The existence of these strains suggests that genetic components exist in the degree and nature of alcohol drinking behaviour of rodents, although it is unlikely that a complex disorder like alcohol addiction could ever be fully explained in terms of the action of a single gene (Holden, 1991). Random bred rats are used to study all kinds of physiological changes coupled with (prolonged) ethanol consumption.

The classical and, at one point, only model of experimental alcohol administration is the incorporation of ethanol in the drinking water of the animals. This technique has the advantage of simplicity, but it has two drawbacks, namely: the nutritional effects associated with the consumption of alcohol are difficult to assess and to control; the alcohol intake is low, as a result of the natural aversion of most animals for ethanol. When the diet is adequate, no significant liver damage is caused (Best *et al.*, 1949).

Today, three categories of animal models can be recognized, each concerned with a different aspect of ethanol consumption. To study alcohol addiction an important criterion for experimental models is that animals have to self-administer alcohol, they must have a wish to consume alcohol (Stewart & Grupp, 1989). This forces the use of self-administration models like schedule-induced polydipsia (rats maintained on a fixed interval schedule of reinforcement for food consumed copious amounts of alcohol during the intertrial intervals), preference studies or operant studies (Kornet, 1991).

Physiological complications observed in alcoholics can be reproduced with the liquid diet technique (DeCarli & Lieber, 1967; Lieber & DeCarli, 1982). This diet induces fatty liver, hyperlipemia, various metabolic and endocrine disorders, tolerance to ethanol and other drugs, physical dependence and withdrawal and, in the baboon, liver fibrosis and cirrhosis. Main disadvantage is that control and experimental animals have different feeding patterns. One day the

total caloric intake of the ethanol consuming rats is measured. The other day the control rats get the same amount of calories without ethanol. Control animals consume the major portion of their food on initial feeding and effectively starve themselves for the remainder of the 24-hr period (Keane & Leonard, 1989).

Alternatively the alcohol-drinking water techniques is used. This is more appropriate if one is interested primarily in more specific effects of ethanol, and if the effect is observed with lower levels of ethanol consumption. The ethanol-drinking water technique provides a simple and useful tool, as illustrated by the induction of a specific microsomal cytochrome P450 in the liver (Lieber *et al.*, 1989). Both the Lieber diet and the ethanol-drinking water technique are isocaloric, not isonutrient.

3. POLYCYCLIC AROMATIC HYDROCARBONS

3.1 Human exposure

The history of the toxicology of polycyclic aromatic hydrocarbons (PAHs)[1] is closely related to the establishment of the carcinogenicity of coal tar (Phillips, 1983). As early as 1775, the British surgeon Sir Percivall Pott published studies which indicated increased incidences of scrotal cancer among chimney sweeps in England, resulting from prolonged contact of the skin with coal soot (Pott, 1775).

In the nineteenth century, high incidences of skin cancer were reported among workers in the paraffin refining (von Volkmann, 1875), shale oil (Bell, 1876) and coal tar industries (Butlin, 1892). Early attempts to produce cancer in experimental animals with the raw materials of these industries were unsuccessful and it was only in 1915 that investigators succeeded in inducing tumors with coal tar. Yamagiwa and Ichikawa showed that if coal tar was repeatedly painted inside the ears of rabbits (two to three times a week), malignant tumors developed at the site of the skin painting after 3 months, and these later grew over the whole ear (Yamagiwa *et al.*, 1915). Soon afterwards Tsutsui obtained malignant skin tumours in mice by painting them with coal tar (Tsutsui, 1918). Attempts were started to characterize the carcinogens in coal tar. It was found that the carcinogenic factor was concentrated in the high-boiling fractions and was free of arsenic, nitrogen and sulphur (Bloch *et al.*, 1921).

In 1922, Kennaway and his group at the Research Institute of the Cancer Hospital in London started a series of chemical and physical studies of coal tar. It was found that the fluorescence spectrum of benzanthracene was very similar to those of the carcinogenic tars, although shifted to longer wavelengths (Hieger *et al.*, 1930; Kennaway & Hieger, 1930). After the synthesis of dibenz(a,h)anthracene and its 3-methyl-derivative these compounds were tested by

[1] *PAH in this context means homocyclic, non-substituted PAH*

skin painting of mice and found to produce tumors. This was the first recognition of the carcinogenic activity of a chemical of defined structure. Hieger and co-workers (1933) initiated a large-scale isolation of the carcinogens(s) in coal tar. Starting with two tons of pitch, they performed a series of fractional distillations, differential extractions, and crystallizations. The various fractions were characterized by their fluorescence spectra and their carcinogenic activity by skin painting on mice. In two years they had isolated about 7 g of a yellow crystalline material which had the correct fluorescence spectrum and exhibited high carcinogenic activity. This material was found to consist of two isomeric compounds, benzo(*a*)pyrene [B(*a*)P] and benzo(*e*)pyrene [B(*e*)P]. Both the synthetic and the isolated samples of B(*a*)P were highly carcinogenic.

In the following years, a large number of PAH compounds were isolated and synthesized, and were tested for carcinogenic effects by skin painting. These studies were the first of a large number of studies of the carcinogenic properties of PAHs. By 1976, more than 30 parent PAH compounds and several hundred alkyl derivates of PAHs were reported to have some carcinogenic effects (Committee on Biologic Effects of Atmospheric Pollutants, 1972; Dipple, 1976). This makes PAHs and their derivatives the largest class of chemical carcinogens known today. Recently, the International Agency for Research on Cancer (IARC) made a critical evaluation of all available data on the carcinogenic effect of 32 PAHs. It was concluded that 11 PAHs were found to be proven carcinogens in experimental animals, 6 were found likely to be carcinogens, 12 had inconclusive evidence, and 3 were proven noncarcinogens (IARC, 1983).

PAHs can be formed by decomposition of any organic materials containing carbon and hydrogen. The formation is based on two major mechanisms: (1) pyrolysis and pyrosynthesis or incomplete combustion, and (2) low temperature carbonization processes. In addition, some investigators suggest a biological formation of PAHs (Bjorseth *et al.*, 1986).

Although the mechanism of PAH formation in combustion processes is complex and variable, Badger and coworkers (1962) suggested the stepwise synthesis of PAHs from C_2 species during hydrocarbon pyrolysis. Once formed, PAHs might undergo further pyrosynthesis reactions to form larger PAHs by intermolecular condensation and cyclization (Lang *et al.*, 1963).

Only a limited number of PAHs enter the environment. Irrespective of the type of material to be burned, surprisingly similar ratios of PAHs are formed at a defined temperature. For example, thermal decomposition of pit coal, cellulose, tobacco, and also of polyethylene and polyvinylchloride carried out at 100 °C yields very similar PAH profiles (Grimmer, 1983). The absolute amount of PAHs formed under defined pyrolysis conditions depends, however, on the temperature as well as the material.

PAHs in fossil fuels have been formed at low temperatures by a carbonization process over millions of years. The PAH profile contained within

fossile fuels is clearly different from that obtained by pyrolysis. In petroleum, the alkylated PAHs outnumber the parent PAHs.

The amount of PAH possibly formed by biosynthesis in microorganisms or plants is negligible in comparison with the quantities formed by abiotic processes (Grimmer, 1976).

Figure 7 lists the names and structures of parent PAHs (with 3 or more rings) most frequently found in environmental samples.

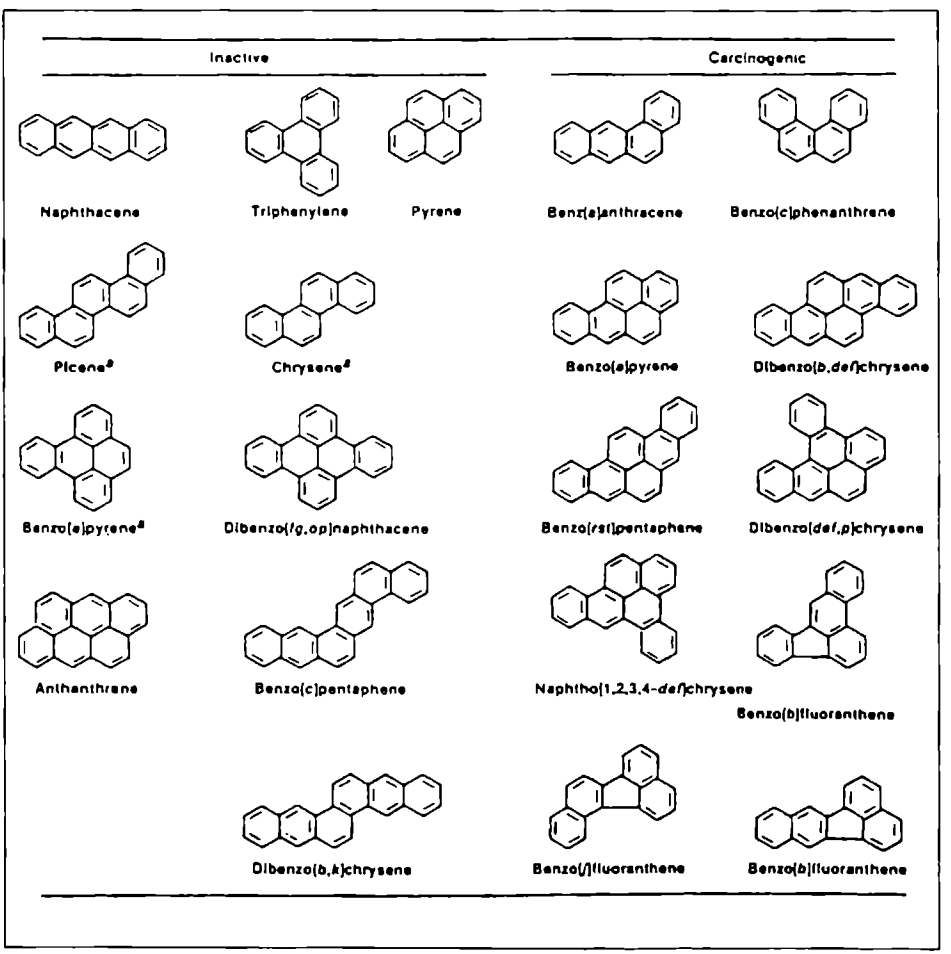


Figure 7. Structures of carcinogenic and non-carcinogenic unsubstituted polycyclic aromatic hydrocarbons (PAHs). [a]These compounds show tumor-initiating activity. From Dipple *et al.* 1984

3.1.1 Environmental exposure

The routes of exposure to PAHs for the general population are through inhalation of air, drinking of water and intake of food. Emissions of PAHs in the air began with forest fires and volcanic eruptions and have greatly increased as consequence of industrialization. In the Netherlands, about 1350 tons of B(*a*)P is produced and/or imported yearly (Verhoeve *et al.*, 1980). The emission is about 10 tons/year. The largest quantity of B(*a*)P comes from the production and import of coal-tar (1250 tons). Of this amount about 75% is transformed into coal-tar pitch (920 tons B(*a*)P) and heavy oil (30 tons B(*a*)P). The largest part of the coal-tar pitch is used by the graphic and electrode-manufacturing industry, a smaller part in the production of active coal. Coal tar pitch is also used in various (industrial) products such as binding agents, roofing products and coatings. Heavy oil is used for the production of soot.

The total PAH emission into the air is several thousands of tons/year. These consist for over 90% of lower PAHs (molecular weight < 200-230) and 80% of these emissions come from industrial sources. PAH emissions into water amount to about 150 tons/year. These also consist mainly of lower PAHs (for over 90%). Diffuse sources are responsible for more than 90% of these emissions (van der Naald *et al.*, 1988).B(*a*)P emissions are mainly produced in industry (65%). Large diffuse sources are the use of coal burning as heating source and waste oil (14% and 18%, respectively).

Almost all PAHs with 4 or more rings present in the air are adsorbed onto particles which are deposited on the ground and in the water and thus contribute to exposure through drinking water and food. The major portion of PAHs in ambient air is found adsorbed on particles smaller than 0.5 μm. About 500 PAHs have been detected in the air but most measurements concern B(*a*)P. B(*a*)P levels in clean air are < 1 ng/m^3 but heavely polluted air can contain levels up to 100 ng/m^3 (Hemminki, 1990). In the Netherlands, the amount of B(*a*)P in the air is usually about 10% of the total amount of all PAHs. The concentration of B(*a*)P in the air, averaged over one year, amounts to 1 ng/m^3 for the past few years; higher concentrations have been found in the IJmuiden-area (Hoogovens): up to 4 ng/m^3. Up to the nineteen sixties these concentrations were much higher: often higher than 20 ng/m^3. Since then the concentrations of B(*a*)P -and presumably all PAHs- have decreased strongly, probably as a result of the large scale use of natural gas as an energy source, instead of coal. Adult humans inhale about 20 m^3 air per day and the PAH deposition rate in lungs amounts to about 50%. This results in intakes between 10 and 1000 ng B(*a*)P per day for clean air. For comparison, the average total B(*a*)P content of the mainstream smoke of one cigarette can vary between 20 and 40 ng.

Smoking and the use of wood in fireplaces cause a considerable increase in the background concentration of PAHs in interior air (Slooff *et al.*, 1989). Data on PAHs in food were reviewed recently (Grasso, 1984; Grimmer, 1983; Vaessen,

1988). PAHs are found in substantial quantities in some foods, depending on the method of cooking, preservation and storage; the B(*a*)P content in charcoal-broiled and smoked foods was reported to be as high as 50 μg/kg. PAHs are detected in a wide range of fresh meat, fish, vegetables and fruits; high levels were found in leafy green vegetables. The latter levels paralleled with the atmospheric burden, indicating that PAHs are deposited from the atmosphere (Grimmer, 1983). It is assumed that 99% of the environmental exposure to PAHs can be attributed to food, 0.9% to inhalation and 0.1-0.3% to the drinking of water (WHO, 1984). A recent study of Dutch total diet showed that the major daily PAH intake comes from sugar and sweets, cereals, oils, fats and nuts and ranged up to 1.4 μg/kg (de Vos *et al.*, 1990).

3.1.2 Occupational exposure

PAHs in the workplace atmosphere may originate from two major processes: (1) evaporation during heating of PAH-containing matter, and (2) formation by pyrolysis and pyrosynthesis or incomplete combustion. Coal tar products, derived from the carbonization of coal, are the most important sources of PAH emissions in the work environment (NIOSH, 1977; Kipling, 1976). Coal tar products are used at elevated temperatures in many industrial processes, such as aluminium melting, iron and steel production, and foundry and asphalt production thereby emitting PAHs into the work atmosphere. In some studies, average daily inhaled doses of 50 μg B(*a*)P have been observed (Hammond *et al.*, 1976).

The main Dutch industries associated with considerable occupational (and environmental) pollution of PAHs are: carbon electrode plants, coke works, a needle coke plant, aluminum producings plants, a coal tar distillation plant, phosphor plant, silicium carbide plant, and an iron and steel works. More peripheral industries are wood preserving industries, soil decontamination of tar-contaminated soil, asphalt handling (re-use of coal tar asphalt), glassworks, foundries. Peripheral exposures are associated with the production, handling and application of: hot tar derived products, tar derived coatings, waste oil, exposure to exhaust gases (industrial + vehicle), carbon black or carbon-black derived materials (Jongeneelen, 1987).

Pathways for exposure of workers to PAHs are the inhalation of vapors or particles, contamination of the skin, and ingestion. The relative importance of each exposure route varies according to the particular technology used or the particular type of work in which the worker is engaged. Exposure to PAH vapors may not pose a direct carcinogenic risk, because only high-boiling PAHs with correspondingly low vapor pressures have been demonstrated to be carcinogenic. However, some of the vapors could exert promotional or co-carcinogenic responses when inhaled together with other vapors or particulate PAHs.

PAHs with four or more rings are primarily associated with particles when they occur in the atmosphere. The particles may be deposited in various compartments of the respiratory tract depending on the physical properties of the particles, the air velocity in the respiratory tract, and the branching of the respiratory tract (Pott & Oberdörfer, 1983). Particles exceeding 10 μm in aerodynamic diameter usually remain in the upper respiratory tract and do not penetrate into the lungs.

In other industries, skin contact with PAH-containing material may be the major route of human exposure to PAHs. As PAHs are highly lipophilic, they readily penetrate the skin and may cause systemic effects.

Direct ingestion of particles will be prohibited by good occupational hygiene and will seldom occur. However, the inhalation and swallowing of coarse particles may represent an indirect route of ingestion. The inhaled larger particles will be trapped in the mucous fluids of the upper and middle respiratory system. After transport to the throat, they will be swallowed.

3.2 Fate in the body

3.2.1 Absorption, distribution and elimination

Mitchell (1982, 1983) showed that 1 to 2 μm aerosol particles of B(*a*)P inhaled by rats were deposited mainly in the upper respiratory tract. B(*a*)P was cleared from the lungs and transported to the internal organs in two phases: an initial rapid phase where 50% of the B(*a*)P was cleared in approximately 2 hr and a second slower phase that continued for about 2 days after exposure. Metabolites of B(*a*)P were found in the lung, liver, and kidney shortly after the inhalation exposure. The amount of unmetabolized B(*a*)P in lung tissue was not greater than 20%. The results indicated that a large portion of inhaled B(*a*)P was metabolized by the lung tissue prior to clearance from the lung. Furthermore, it was found that a significant amount of B(*a*)P was covalently bound to the lung tissue and retained for a much longer time than the soluble metabolites.

Sun *et al.* (1982) found that about 10 % of pure B(*a*)P aerosol (approximately 0.1 μm in diameter) was deposited in the lung of rats. During the exposure, and within the first 30 min after exposure, about 90% of the amount of B(*a*)P was cleared from the lung by absorption into blood followed by excretion from the body through the feces. The retention of B(*a*)P, however, was much longer when B(*a*)P coated on insoluble particles was inhaled. Furthermore, both studies indicated that a substantial amount of inhaled B(*a*)P particles were removed from the lung by mucociliary clearance and subsequent swallowing of the material. This mechanism can result in high PAH levels in the GI tract from which they may be absorbed. This may be a second important route of exposure to PAHs. The lung retention of particle-bound PAHs depends on particle-size, but will always be higher than gaseous PAHs. Small particles penetrate the deeper

parts of the lungs and are retained there for a long time; a relatively large proportion of PAHs will therefore elute at that location. Larger particles stay in the upper respiratory tract, where clearance is more rapid and therefore less PAH elution from the particles occurs. It was estimated that, in man, approximately 20% of the inhaled higher PAHs as occurring in outdoor air will come into contact with lung tissue after retention in the lungs and elution (Van Vaeck & Cauwenberge, 1983).

In a study by Heidelberger and Weiss (1951), topical application of a B(*a*)P solution to shaved backs of mice was followed by a diphasic disappearance of radioactivity, with half-lives of 40 and 104 hr for the initial rapid phase and the subsequent slow phase, respectively. Since essentially all of the radioactivity was recovered in the feces within 16 days, quantitative percutaneous absorption of B(*a*)P is apparent.

PAHs, once absorbed, become localized in a wide variety of body tissues. The distribution of radioactivity derived from ^{14}C-B(*a*)P in the rat and mouse was determined following subcutaneous, intravenous, and intratracheal administration (Kotin *et al.*, 1959). The pattern of distribution was found to be similar in all cases, except for high local pulmonary concentrations following intratracheal instillation. Concentrations of B(*a*)P-derived radioactivity in the liver reached a maximum within only 10 min after injection and represented 12% of the total dose. Radioactivity in the liver was reduced to 1 to 3% of the administered dose within 24 hr. Similarly, maximum blood levels of B(*a*)P following intra venous injection were reached very quickly, and radioactivity became barely detectable after 10 min. Minimal tissue localization of radioactivity (B(*a*)P and/or its metabolites) occurred in the spleen, kidney, lung, and stomach; maximum radioactivity derived from labeled B(*a*)P was recovered in the bile and feces. Levels of radioactivity in fat, skin, and muscle were not determined, nor was the amount of unchanged B(*a*)P in any tissue. Bock & Dao (1961) later showed that, relative to other tissues, unmetabolized B(*a*)P was located extensively in the mammary gland and general body fat after a single feeding of the carcinogen (10 to 30 mg). According to Bjorseth and Becher (1986), the results of the studies indicate the following: 1. Detectable levels of PAHs and/or PAH metabolites can be observed in most internal organs from minutes to hours after administration. 2. Mammary and other fat tissues are significant storage depots where PAHs may accumulate and be slowly released. 3. The gut contains relatively high levels of PAHs and/or PAH metabolites as a result of hepatobiliary excretion or of the ingestion of particulate PAHs following mucociliary clearance after inhalation.

Hepatobiliary excretion and elimination through the feces is the major route by which PAHs are removed from the body. Kotin *et al.* observed that 4 to 12% of the subcutaneously injected dose of B(*a*)P was eliminated in the urine of mice within 6 days after injection, while 70 to 75% of the dose was recovered in the feces. Camus *et al.*, 1984) determined fecal excretion rates of B(*a*)P in two

different strains of mice following i.p. injection of B(*a*)P. The cumulative excretion of B(*a*)P in the feces followed an exponential curve with half-lives of 1.2 and 0.6 days for the two different strains. The majority of metabolites in the urine appeared as highly water-soluble conjugates. After enzymatic deconjugation, various oxidated B(*a*)P metabolites could be identified.

Fecal excretion is the major pathway for the elimination of B(*a*)P and metabolites from the body. Depending upon the dose given, a nonabsorbed portion of the xenobiotic contributes to fecal excretion. The part of the dose that is absorbed from the gastrointestinal tract in the blood passes through the liver before reaching the general circulation. The liver is the main site of biotransformation of B(*a*)P and the metabolites thus formed may be excreted directly into bile. B(*a*)P and/or metabolites entering the intestine with bile may be excreted with feces, or when the physicochemical properties are favorable for reabsorption, an enterohepatic circulation may ensue. Once a compound is excreted into bile and enters the intestine, it can either be reabsorbed or eliminated with feces. Conjugated polar metabolites are not sufficiently lipid soluble to be reabsorbed. However, intestinal microflora may hydrolyze glucuronide and sulfate conjugates. Reabsorption of a xenobiotic completes an enterohepatic cycle. An increase in hepatic excretory function has been observed after pretreatment with some drugs but B(*a*)P is relatively ineffective (Klaassen & Rozman, 1991).

3.2.2 Metabolism of PAHs

PAHs themselves are not chemically reactive, but they exert their carcinogenic activity through metabolites which are sufficiently reactive to modify cellular macromolecules such as nucleic acids (DNA, RNA) and proteins (Miller & Miller, 1973). Metabolism is dominated by oxidation through the microsomal cytochrome P450 dependent mixed-function oxidase (MFO) system, also termed aryl hydrocarbon hydroxylase (AHH) (Nebert & Gonzalez, 1987). The initial step in the metabolism of unsubstituted PAHs is the formation of epoxides involving the addition of oxygen across a double bond. Further metabolism of epoxides results in hydration to dihydrodiols by epoxide hydrolase, isomerization to phenols, or conjugation with glutathione by glutathione S-transferase. Glutathione conjugates can be easily excreted as mercapturic acids in urine but the phenols and dihydrodiols are often not sufficiently polar to be excreted as such. These hydroxylated metabolites are conjugated with glucuronic acid or sulphuric acid to facilitate excretion. The enzymes that catalyze these conjugations are glucuronyl-transferases, located in the endoplasmic reticulum, and sulphotransferases which are cytosolic enzymes (figure 8).

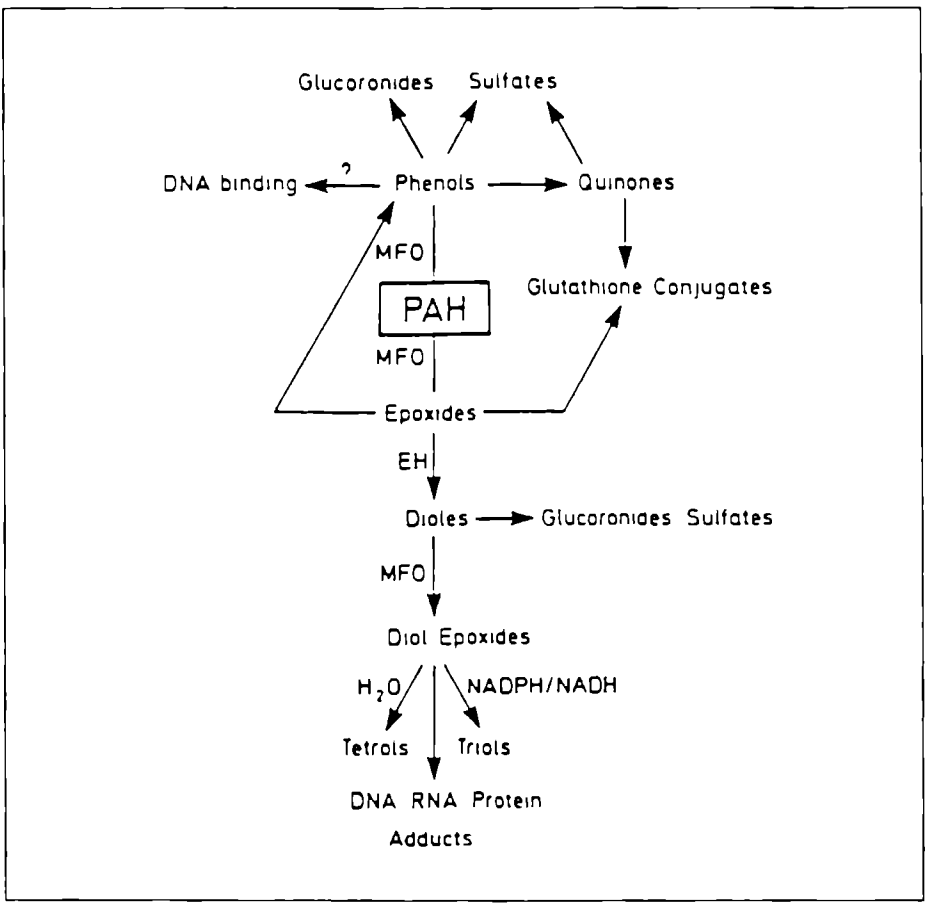


Figure 8 Enzymatic pathways involved in the activation and detoxification of PAHs

The metabolism of PAHs results mainly in the formation of more water soluble derivatives which can be excreted. Sometimes, however, the enzyme system will activate the parent compound into biologically reactive forms.

Certain 'bay region diol epoxides' are the main DNA binding metabolites of B(*a*)P (Sims *et al.*, 1974; Grover *et al.*, 1967). Fluorescence spectral evidence was found that benzo(*a*)pyrene-DNA products in mouse skin arise from diol-epoxides (Daudel *et al.*, 1975). Both B(*a*)P-7,8-epoxide and B(*a*)P-7,8-dihydrodiol exist as pairs of enantiomers. Consequently, two diastereoisomeric vicinal B(*a*)P-7,8-dihydrodiol 9,10 epoxides (BPDE's) exist as

either the *syn-* or *anti*-form [the epoxide oxygen on either the same *(syn)* or the opposite *(anti)* face of the ring as the hydroxyl group in the 7-position], each comprising two enantiomers. In fact, the monooxygenases involved are highly stereoselective and the addition of oxygen across the 7,8 double bound results predominatly (>90%) in the (+)-7**R**,8**S**-epoxide and the (-)-7**R**,8**R**-dihydrodiol, whilst further oxidation of the dihydrodiol yields mainly (+)-*anti*-7,8-dihydrodiol-9,10-epoxide from the **R,R** enantiomer and mainly (+)-*syn*-7,8-dihydrodiol-9,10-epoxide from the **S,S** enantiomer (Dipple *et al.*, 1984; Yang *et al.*, 1984) (Figure 9).

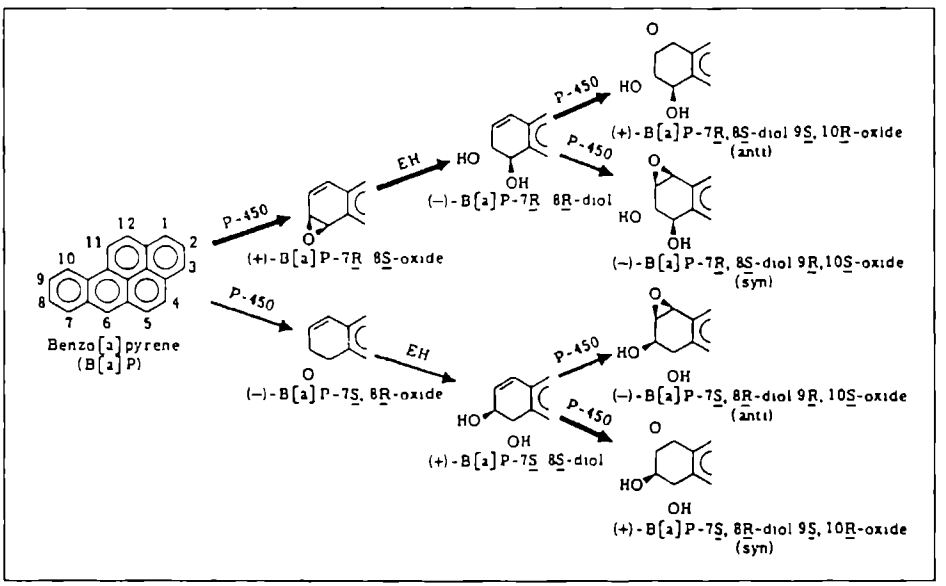


Figure 9 Stereochemistry of the metabolic activation of benzo(*a*)pyrene [B(*a*)P].

The four resulting stereoisomers differ markedly in their biological reactivity, and in most test systems (+)-*anti*-B(*a*)P-7,8-dihydrodiol-9,10-epoxide has been found to possess greater activity than the other three metabolites (Dipple *et al.*, 1984). DNA adducts formed in tissues or cells treated with B(*a*)P are derived predominantly from the (+)-*anti*-dihydrodiol-epoxide, with minor involvement of the (+)-*syn*-dihydrodiolepoxide in some cases (Weston *et al.*, 1983). In DNA and polyribonucleotides, B(*a*)P dihydrodiol-epoxides react principally with guanine. The major adduct is (+)-*anti*-BPDE-dG and the linkage is between the 2-amino group of guanine and the C-10 position of B(*a*)P (Weinstein *et al.*, 1976). The

dihydrodiol epoxides can also bind to the N-7 of guanine, the exocyclic amino groups of adenine and cytidine and to phosphate residues. It should be mentioned that other metabolites of B(*a*)P, for example B(*a*)P-4,5-oxide, can also bind to DNA, especially under *in vitro* conditions (Baird *et al.*, 1975).

3.3 Health effects of PAHs

3.3.1 Cancer

Excess of lung cancers was found in the British and Japanese gas works (Doll *et al.*, 1972; Kawai *et al.*, 1967), in the American, Japanese and British coke works (Lloyd, 1971; Redmond *et al.*, 1976; Sakabe *et al.*, 1975; Mazumdar *et al.*, 1975) and in the Russian, Canadian and American aluminium industry (Konstantinov *et al.*, 1979a,1979b; Gibbs, 1979,1985; Aluminium Ass, 1977). An investigation of 2071 chimney sweeps also showed an excess of lung cancer (Hogstedt *et al.*, 1982). Metal workers exposed to coal tar pitch volatiles showed a two-fold increase of lung cancer (Silverstein *et al.*, 1985).

In the past, scrotum cancer cases have been reported from chimney sweeps (Pott, 1775; Paget, 1850). An excess of skin cancer was found in the Russian aluminium industry (Konstantinov *et al.*, 1979a) and among workers handling hot pitch and asphalt (Hammond *et al.*, 1976).

Many authors have found an increased incidence of bladder cancer among coke oven workers. Generally, it is the presence of certain amines that is held responsible for this particular type of cancer (Zorn, 1966). An increase of cancer of the gastro-intestinal tract was found in American coke works and among workers handling pitch and asphalt (Redmond *et al.*, 1976; Hammond *et al.*, 1976). Excess malignant neoplasms of the stomach and oesophagus were found among aluminium reduction plant workers (Gibbs, 1985). Metal workers exposed to coal tar pitch volatiles (CTPV) showed a two-fold excess of cancer of the digestive organs (Silverstein *et al.*, 1985). Workers handling pitch and asphalt showed an increase of leukaemia (Hammond *et al.*, 1976). A slight and non significant increased frequency of leukaemia was observed among aluminium industry workers (Aluminium Ass, 1977). Metal workers exposed to CTPV showed a four-fold excess of leukaemia (Silverstein *et al.*, 1985).

Non-carcinogenic effects of PAHs-containing coal tars and creosote on humans have also been studied. The majority of toxic effects concern the skin and eyes; skin effects include dermatitis, chronic tar dermatosis, tar or pitch warts, cutaneous phototoxicity and chronic melanosis, folliculitis and pitch acne (IARC, 1986). Naphthalene and anthracene are both primary (mild) irritants. High naphthalene concentrations causes cataract, whereas anthracene and phenanthrene have photo-sensitizing properties. Only little information is available about the effects on reproduction and foetal/embryonal development.

Epidemiological evidence suggests that smoking of tobacco is causally related to cancer of the respiratory tract, the upper digestive tract, pancreas, renal pelvis, bladder, and cervix (IARC, 1986). It has been estimated that more than 80% of deaths from lung cancer and 30-40% of deaths from all cancers can be attributed to smoking (Loeb *et al.*, 1984; IARC, 1986). In addition to active smoking, passive smoking i.e. exposure to side stream tobacco smoke or environmental tobacco smoke, has been incriminated as a risk factor for cancer of the lung in nonsmokers (IARC, 1986; Saracci & Riboli, 1988).

More than 3800 chemicals have been identified in tobacco smoke comprising at least 40 known carcinogens including tobacco-specific nitrosamines, aromatic amines and PAHs (reviewed in IARC, 1986; Hoffman & Hecht, 1990). Bos & Henderson (1984) identified 70 genotoxic agents in main stream cigarette smoke, among which 23 PAHs. Animal studies showed the major tumorigenic activity of tobacco smoke to be concentrated in the particulate matter (tar), whereas for the gas phase no tumorigenic acitivity has been established, although it contains traces of carcinogens. The contribution of tar to the carcinogenic action of cigarette smoke was also shown in epidemiological studies which found cigarettes with low tar yields to be less hazardous than high tar cigarettes (Wynder & Hoffmann, 1982). Although the molecular events associated with cigarette smoking-induced carcinogenesis are not definitively identified, it is thought that they are related in part to the genotoxic activities of the chemicals associated with cigarette smoking (De Marini, 1983). In addition, there is evidence for the presence of compounds in cigarette smoke that can act as tumor promoters or cocarcinogens (Willey *et al.*, 1987).

Studies with laboratory animals have demonstrated a possible role for PAHs as tumor initiators in tobacco-related carcinogenesis. For instance, several studies showed the occurrence of respiratory tract tumors in Syrian golden hamsters after inhalation of B(*a*)P (Thyssen *et al.*, 1981), intratracheal instillation of PAHs (Saffioti *et al.*, 1985), and intratracheal instillation of 7,12-dimethylbenzanthracene, followed by exposure to tobacco smoke (Kobayashi *et al.*, 1974). Additional evidence for a role of PAHs in tobacco-related carcinogenesis came from bioassays on mouse skin using subfractions of the particulate matter containing PAHs (Hoffman *et al.*, 1978). PAHs also induced lung tumors in rats upon implantation of PAHs in the lung (Deursch-Wenzel *et al.*, 1983).

3.3.2 Mechanisms of carcinogenic effects of B(a)P

The International Agency for Research on Cancer (IARC, 1983) has stated that there is sufficient evidence that 11 PAHs are carcinogenic to experimental animals. However, human carcinogenicity data are only available for PAH mixtures, mostly including other substances as well.

The formation of DNA adducts is believed to be a necessary step in the process by which these carcinogens exert their biological effects. Alteration of DNA, if not recognized and removed by DNA repair processes, may result in changes of the genome through point mutations, deletions, gene amplifications or rearrangements (Singer & Grunberger, 1983). Point mutations but also other events may lead to the activation of proto-oncogenes (Stowers *et al.*, 1987). At present, it is known that several types of tumors induced in rodents by chemical carcinogens and certain tumors in humans are associated with base substitutions at specific sites in the *ras* oncogene family (Bos, 1989). The activation of proto-oncogenes to oncogenes results in altered levels of expression of normal proteins, or in expression of abnormal proteins (Weinstein, 1988). The number of proto-oncogenes that must be activated in order to convert a normal cell into a malignant one is currently known for certain tumors and both activation of proto-oncogenes and inactivation of tumor suppressor genes are involved.

3.3.3 Human risk assessment

In 1984 the Health Council of the Netherlands stated that no toxicological limit values could be given for ambient air because the concentration range of human exposure was too small compared to the doses used in experimental animals (Gezondheidsraad, 1984). In 1989 an integrated criteria document about PAHs was produced by the National Institute of Public Health and Environmental Protection in which toxicological limit values were given.

Attempts for risk extrapolations for the general population were made by Kramers and van der Heijden (1988). For the oral route the authors relied on animal data. Chronic studies with B(*a*)P were available, although none of these met the criteria currently applied to this type of study (Rigdon & Neal, 1966; Neal & Rigdon, 1967; Horie *et al.*, 1965; Chouroulinkov *et al.*, 1967). Based on the studies of Horie and of Chouroulinkov, Gold *et al.* (1984) calculated a TD50 value for B(*a*)P of 1.86-12.4 mg/kg body weight/day; i.e. this daily dose given lifetime would produce tumours in 50% of the animals. Estimates of daily uptake of B(*a*)P via food consumption amount to 0.16-1.6 μg in the US (Santodonato *et al.*, 1981), whereas the levels as measured by Vaessen *et al.* (1987,1988) in duplicate diets in the Netherlands average 0.08 μg per person per day. Kramers and van der Heijden (1988) carried out a linear non-treshold extrapolation from the TD50 value. They calculated that the daily intake of 0.08 μg B(*a*)P would represent an extra lifetime tumour risk of 0.06-0.4 per million for only B(*a*)P. This might be multiplied by a factor of 25 to account for all carcinogenic PAHs, yielding a PAH risk of 1.5-10 per million. According to the authors, this range is insufficient considering the insecurity of this kind of operation. The studies by Rigdon and Neal (1966) and Neal and Rigdon (1967) produced data suggestive of a concave exposure-response

relationship. Besides, more than half of the tumours on which the calculated TD50 value was based were in fact non-malignant papillomas of the forestomach.

Concerning exposure via air, the WHO working group on Air Quality Guidelines (1986) has recently provided a risk estimate for PAHs in ambient air, on the basis of epidemiological data collected from cohorts of coke-oven workers by Redmond *et al.* (1976) and a calculation made by EPA (1984) using these data. With a linearized multi-stage model, EPA has calculated an excess risk of 6.2×10^{-4} at lifetime exposure to 1 $\mu g/m^3$ of the fraction of coke-oven emission which is defined by its solubility in benzene. Taking a B(*a*)P content of 0.71% of this fraction (WHO, 1986), this would convert to a risk of 8.7×10^{-5} after lifetime exposure to 1 ng/m^3 B(*a*)P plus other carcinogens present in coke-oven emissions. This is the same value as presented in the dutch Integrated Criteria Document on PAHs. Average B(*a*)P concentrations in dutch ambient air range from 0.3 to 0.7 ng/m^3, depending on the location (Thijsse & Huygen, 1985) but in certain areas and/or periods the level often exceeds 1 ng/m^3. The upper limit B(*a*)P levels (plus other PAHs) would present an individual lifetime risk of 15-60 per 10^6.

Risks due to food versus ambient air have been compared. The maximum B(*a*)P uptake (assuming 100% uptake of PAHs from inhaled air and 15 m^3 of air inhaled per 24 h (Spector, 1956) for the average levels of 0.3-0.7 ng/m^3 B(*a*)P would amount to 4.5-10.5 ng/day, being in the order of 10-fold less than the estimated uptake via food (0.08 μg B(*a*)P/day). Therefore, we may consider food as the main source of the human PAH burden. However, the risks as calculated above, in connection with the exposure levels, rather suggest the opposite, the inhalation risk exceeding the one for food uptake. This is due to the difference in the effective dose of the reactive metabolites at the target level (DNA), caused, for instance, by differences in metabolism and repair capacity in the affected tissues.

3.4 Biomarkers of PAH exposure

In the past, most knowledge on PAH toxicokinetics has been obtained by using radiolabeled compounds in experimental animals. Nowadays, reversed-phase HPLC on chemically bonded nonpolar phases (such as C_{18}) is the most popular LC method for the separation of PAHs (metabolites). A major advantage of HPLC for the determination of PAHs is the availability of sensitive and selective detectors. When a HPLC system is connected to a fluorescence spectrofotometer, several metabolites can be detected without the use of radioactive labelled B(*a*)P. The UV detector operated at 254 nm is universal for PAHs, as all PAHs exhibit some absorption at this wavelength. Fluorescence detection of PAHs provides much greater sensitivity and selectivity than UV detection. The selectivity for individual PAHs (metabolites) is optimized by changing the fluorescence excitation and

emission wavelengths during the reverse-phase chromatographic separation (Bjorseth & Becher, 1986).

To evaluate exposure and health risks in humans, methods for biological monitoring (BM) and biological effect monitoring (BEM) have been implemented besides environmental monitoring (EM) approaches.

Environmental monitoring agents at the workplace or in the natural environment are measured to evaluate the ambient exposure and to assess the health risk. The most widely used method for air pollutants containing PAHs is the measurement of the benzene or cyclohexane soluble fraction of suspended particulate matter (NIOSH, 1977). A second method is the determination of a single PAH, for instance B(*a*)P, in airborne particulate matter. A third method is the measurement of a group of PAHs, for example the 6 Borneff components (Borneff & Kunte, 1969) or the 16 EPA-components (EPA, 1979) or a complete analysis. In several countries treshold limit values are based on one of these measurement methods.

Biological monitoring is primarily an activity during which repetitive measurements of toxic chemicals in biological specimens are applied to assess the exposure levels of individual workers and of groups of workers. Four ways of biological monitoring of PAHs have been proposed: the urinary mutagenicity assay, reduction of urinary PAH-metabolites to their parent compounds, determination of the unmetabolized B(*a*)P in body fluids and determination of a representative metabolite for exposure to complex PAH mixtures.

The prevalence of different biological effects in PAH-exposed people was reported and can be considered as possibilities for monitoring; the determination of B(*a*)P-DNA-adducts in peripheral blood lymphocytes, sister chromatid exchanges in blood lymphocytes, chromosomal aberrations in cultured lymphocytes from blood samples and semen abnormalites (Jongeneelen, 1987). Since the formation of specific DNA modifications appeared to be a critical event in carcinogenesis, measurement of carcinogen-DNA adducts should provide biologically relevant information on the net result of exposure, absorption, metabolism, DNA adduct formation and DNA repair in man and experimental animals.

^{32}P-postlabelling analysis is currently the most sensitive technique available for the detection of aromatic adducts. Only small quantities (1-5 μg) of DNA are required to perform the analysis. The covalent binding of a number of PAHs to DNA *in vivo* has been analyzed by ^{32}P-postlabelling (Phillips, 1990a). Each PAH that binds to DNA produces a characteristic pattern of spots on PEI-cellulose TLC (Reddy *et al.*, 1984). A major advantage of the postlabelling assay is the detection of unidentified adducts. In most cases in which standards were available, such as BPDE-DNA adducts, it has been found that nearly quantitative labelling of adducts can be achieved (van Schooten, 1991). The value

of this approach to estimate intake levels and consequent damage of health has not been proven yet.

4. TOXICOLOGICAL INTERACTIONS

Chemical mixtures and combined exposures are aspects of human risk assessment which need to be considered. Man can be exposed to various combinations of chemicals including drugs, ethanol, cigarette smoke, dietary constituents, and environmental pollutants. Interactions between these chemicals have to be taken ino account while evaluating the safety of the individual xenobiotics. Interactions between chemicals can result in toxic effects quite different from the simple sum of the effects of the individual compounds. Joint exposure can give rise to additive, synergistic (more than additive) or antagonistic (less than additive) effects. Interaction can take place in the exposition phase, in the pharmacokinetic (absorption, distribution, metabolism, excretion) phase and in the toxicodynamic phase (toxic effect at target organ).

4.1 Pharmacokinetic interactions between alcohol and drugs

The effect of ethanol on the metabolism of drugs, and vice versa, can be partly explained by their common oxidative pathway through the cytochrome P450 system. Other interactions can be mediated by alteration of ADH, AlDH, gastric emptying, plasma binding of proteins and acetylation (Vrij-Standhardt, 1991).

Long-term ethanol consumption results in proliferation of smooth hepatic endoplasmic reticulum and an increase in the level of cytochrome P450 (Iseri *et al.*, 1966; Rubin & Lieber, 1968; Joly *et al.*, 1973). In alcohol consumers, the microsomal ethanol oxidizing system (MEOS) or more specifically P450 2E1 is induced and thus capable of metabolizing larger quantities of its substrates. When alcohol users are sober, this enhanced capacity is used to convert drugs and other xenobiotics more rapidly. If these substances themself are bio-active, they are consequently less effective, but the concentration of metabolites, which are quite often hepatotoxic, increases (Lieber, 1983; Tsutsumi *et al.*, 1990). If a person is not sober the MEOS is blocked by ethanol, the 'preferred' substrate, which causes a delay in elimination and an increased activity of parent drugs as shown in figure 10 (Hasamura & Takeuchi, 1978; Lieber, 1973; Rubin & Hutterer, 1968; Lieber, 1990).

Induction of P4502E1 may also result from the use of certain types of drugs such as barbiturates. Pretreatment with these drugs may activate ethanol metabolism whereas the presence of drugs may have an inhibitory effect.

Although acute and chronic forms of alcohol consumption have a strong effect on drug metabolism, the reverse effect is much smaller (Lieber, 1983).

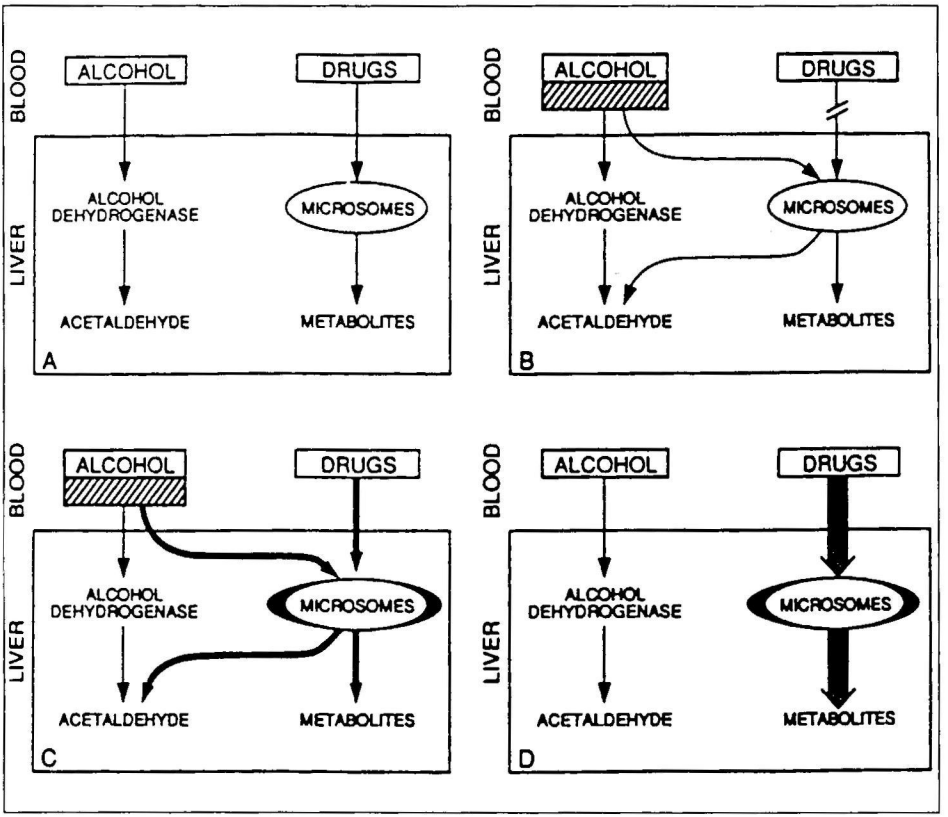


Figure 10. Alcohol is metabolized by alcohol dehydrogenase, and drugs by microsomes. Microsomal drug metabolism is inhibited in the presence of high concentrations of ethanol, in part through competition for a common microsomal detoxification process (B). Microsomal induction after long-term alcohol consumption contributes to accelerated ethanol metabolism at high blood ethanol levels (C). Increased drug metabolism and activation of xenobiotics (due to microsomal induction) persists after cessation of long-term alcoholconsumption contributes to accelerated ethanol metabolism at high blood ethanol levels (C). Increased drug metabolism and activation of xenobiotics (due to microsomal induction) persists after cessation of long-term alcohol consumption (D). Hatching indicates high blood alcohol levels (from Lieber, 1988).

The interaction between ethanol and drugs does not take place via the P450 pathway only (Lieber, 1990). Some antimicrobial drugs inhibit AlDH, resulting in disulfiram-like reactions when combined with ethanol. The effect of drugs converted by ADH can be influenced by ethanol through direct competition. Gastric ADH is inhibited by H2-receptor antagonists such as cimetidine, resulting in higher blood alcohol levels (Caballeria *et al.*, 1989). Alcohol consumption might alter drug absorption by increasing solubility (Linnoila *et al.*, 1979) or by interfering with gastric emptying. Liver injury is usually accompanied with a diminished capacity for and a consequent delay in biotransformation (Klinger, 1981). Alcoholic cirrhosis causes reduced plasma binding of some drugs, resulting in toxic effects, even at therapeutic plasma concentrations. The metabolic rate of drugs metabolized through acetylation is increased by ethanol (Olsen & Morland, 1978) due to enhanced acetyl-CoA production during alcohol metabolism.

There are few data about effects of ethanol exposure on PAH toxicokinetics in man. Considerable PAH intake is seen at occupational sites. A study with coke oven workers (Jongeneelen *et al.*, 1990) showed the effect of the job category at the coke oven, of smoking habits, and of using protective devices on the bodyburden of coke oven workers. A modifying effect of alcohol consumption and the use of medication on the toxicokinetics of pyrene was not established. In this study 1-hydroxypyrene has been suggested as a suitable biological indicator of the internal dose of PAHs (Jongeneelen *et al.*, 1990).

4.2 Cancer risk from joint consumption of alcohol and tobacco

The perhaps most common joint exposure in the daily environment is that of alcohol consumption and tobacco smoking. Rothman and Keller (1972) and Rothman (1976) reanalysed the information on consumption of alcohol and tobacco obtained by Keller and Terris (1965) in their study on US veterans. The analysis showed a synergistic effect between alcohol and tobacco in the development of oral cavity and pharyngeal cancer. Heavy drinkers who were also heavy smokers had a Relative Risk of 15.6 when compared with persons who neither smoked nor drank. These results are in agreement with the findings of Wynder *et al.* (1975a), while Graham *et al.* (1977) found an additive effect of smoking and drinking. In the study of Tuyns *et al.* (1988) a synergistic effect of alcohol and tobacco use on the risk of hypopharyngeal/epilaryngeal cancer was observed.

In the induction of laryngeal cancer, some investigators have reported synergism between alcohol and tobacco (Hinds *et al.*, 1979; Zagraninski *et al.*, 1986). In the study of Tuyns *et al.* (1988), a synergistic model provided an adequate description of the data.

The joint actions of alcohol and tobacco and of alcohol and nutrition on oesophageal cancer have been the subject of several epidemiological investigations. In their studies in north-western France, Tuyns *et al.* (1977,1979) found a synergistic combined effect of alcohol and tobacco, which they described as multiplicative.

Hirayama (1981) found an interaction between tobacco smoking and alcohol drinking in the causation of primary liver cancer. The rate ratios between daily drinkers and other males were 0.9 among nonsmokers, 1.3 among smokers of up to 200,000 cigarettes (life time), 1.2 among smokers of 200,000-400,000 cigarettes per life time, and 1.5 among smokers of more than 400,000 cigarettes. Austin *et al.* (1986) found no interactive effect of tobacco and alcohol consumption on the risk for hepatocellular carcinoma.

4.3 Modifying effects of ethanol on the activity of known carcinogens

N-nitrosodimethylamine (NDMA):
In a study by Griciute *et al.* (1981) in animals given NDMA plus ethanol, several animals developed olfactory tumours that infiltrated the frontal lobe of the brain; no such tumour was observed in animals given only NDMA. No significant difference in the incidence of other tumours was observed. No differences between groups with and without ethanol administration were found in studies by Litvinov *et al.* (1986a) and Teschke *et al.* (1983).

N-nitrosodiethylamine (NDEA):
Griciute *et al.* (1984) found the incidence of malignant oesophageal/forestomach tumours to be higher in the NDEA plus ethanol group than in the NDEA group. Litvinov *et al.* (1986) found an increased incidence of pulmonary tumours. The combination of NDEA plus ethanol increased the incidence of papillomas in the oesophagus and/or forestomach. In contrast, the incidence of epidermoid carcinomas of the oesophagus and/or forestomach was increased only in the high dose group in a study by Gibel (1967).

Other *N*-nitroso compounds:
In studies of Griciute *et al.* (1982,1987) a higher tumour incidence was seen in mice given *N*-nitroso di-n-propylamine and ethanol.
In studies with *N*-nitrosomethylbenzylamine (NMBzA) (Gabrial *et al.*, 1982), *N*-nitrosomethylphenylamine (NMPhA) (Schmähl, 1976), *N*-nitrosopiperidine (NPIP) (Konishi *et al.*, 1986), *N,N'*-dinitrosopiperazine (DNPIP) (Gibel, 1967), *N'*-nitrosonornicotine (NNN) (Castonguay *et al.*, 1984; McCoy *et al.*, 1981), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Takahashi *et al.*, 1986), *N*-hydroxy-2-acetylaminofluorene (OH-AAF) (Yamamoto *et al.*, 1967),

7,12-dimethylbenz[a]anthracene (DMBA) (Kuratsune *et al.*, 1971) no significant treatment-related increase in tumour yield was observed after co-administration with ethanol.

1,2-dimethylhydrazine (DMH):
With DMH ambiguous results were obtained. In the studies of Seitz *et al.* (1984, 1985) a higher number of rectal tumours was seen in rats treated with DMH and ethanol but no difference was seen in tumour incidences at other sites in the intestine. The studies of Howarth & Pihl (1985) and of Nelson & Samelson (1985) showed no significant difference in the incidence of intestinal cancer in the groups given ethanol solution or beer as compared to those given water.
N-nitrosopyrrolidine (NPYR):
A study of NPYR and ethanol in Syrian golden hamsters showed an increased incidence of nasal cavity tumours and tracheal tumours (McCoy *et al.*, 1981). In a subsequent experiment an increased incidence of hepatic neoplastic nodules was observed (McCoy *et al.*, 1986).

Azoxymethane:
The carcinogenicity of azoxymethane was studied by Hamilton *et al.* (1987a,1987b) in Fisher 344 rats. They found the number of right colonic tumours inversely correlated with ethanol consumption in all animals, but no correlation was found with left colonic tumours. The total number of colonic tumours in the high-dose ethanol group was markedly reduced in comparison with controls but not in the low-dose ethanol group. Similar effects were found in the group given 23% calories as beer. In a further study the incidence of tumours of the left, right and transverse colon were significantly reduced by the 33% liquid-ethanol diet given prior to and during administration of the carcinogen.

N-nitrosobis(2-oxopropyl)amine (NDOPA):
NDOPA and ethanol were studied in Syrian hamster by Tweedie *et al.* (1981). Histopathological examination of the exocrine pancreas showed fewer neoplastic lesions in animals treated with NDOPA plus ethanol compared with those treated with NDOPA alone. In another study, Pour *et al.* (1983) found no significant difference in the incidence of pancreatic tumours.

Vinyl chloride (VC):
Radike *et al.* (1981) studied vinyl chloride (VC) in Sprague-Dawley rats that received 5% ethanol in water or water alone as the drinking fluid for life. Rats exposed to VC plus ethanol had more hepatocellular carcinomas and more liver angiosarcomas.

4.4 Modifying effects of ethanol on the toxicokinetics of xenobiotics

The induction of cytochrome P450 by ethanol is associated with an increase in metabolism and toxicity of several xenobiotics, including *N*-nitrosodimethylamine (Garro *et al.*, 1981; Olson *et al.*, 1984), acetaminophen (paracetamol, Strubelt *et al.*, 1978; Sato *et al.*, 1981; Walker *et al.*, 1983), carbon tetrachloride (Hasumura *et al.*, 1974), aflatoxin B_1 (Glinsukon *et al.*, 1978), benzene (Nakajima *et al.*, 1985) and benzo(*a*)pyrene (Seitz *et al.* 1978; Murphy and Hecht, 1986). The unique cytochrome P450 isozyme (IIE1) induced by ethanol catalyses the oxidation of some alcohols, aniline and *N*-nitrosodimethylamine (Yang *et al.*, 1985a,b; Morgan *et al.*, 1981; Villeneuwe *et al.*, 1976).

In contrast, ethanol inhibits the metabolism of xenobiotics *in vitro* like benzene, styrene and toluene (Sato, *et al.*) and benzo(*a*)pyrene (Rubin and Lieber, 1968). Acute administration of ethanol also inhibits the metabolism of some drugs *in vivo* (Rubin *et al.*, 1970; Mezey, 1976; Sato *et al.*, 1985). There are two possible explanations for this phenomenon; direct competition for cytochrome P450 (Sato, *et al.*, 1981) or a decrease in the supply of a cofactor for the monooxygenase system (Reinke *et al.*, 1980).

The hepatic enzymes that catalyse conjugation of xenobiotics, such as UDP-glucuronyl-transferase (Sweeny & Reinke, 1987) and glutathione-S-transferases, are reported to be induced by feeding ethanol (Schnellmann *et al.*, 1984). Glucuronidation was inhibited by concomitant ethanol administration in isolated hepatocytes and in rat liver microsomes (Sundheimer & Brendel, 1984). Glucuronidation and sulphation were inhibited in perfused rat liver (Reinke *et al.*, 1986).

Pretreatment of rats with ethanol increases the metabolism of *N*-nitrosamines in the lung, oesophagus (Farinati *et al.*, 1985) and nasal mucosa (Castonguay *et al.*, 1984). This contrasted with the observations when ethanol was given concomitantly with NDMA. Concentrations of the unmetabolised nitrosamine in brain, lung, liver, kidney and blood were then increased markedly (Anderson *et al.*, 1986). First-pass clearance of NDMA by the liver in rats was prevented when it was administered in ethanol (1 ml, 5% v/v) instead of in water. The prevention of first-pass clearance had a dramatic effect on alkylation of kidney and oesophageal DNA: at dose levels of 30 μg/kg body weight NDMA, methylation of kidney DNA of ethanol-treated rats was five times that in controls; lower doses of NDMA induced methylation in the kidneys of ethanol-treated rats but not in controls (Swann, 1984). Similarly, administration of *N*-nitrosodiethylamine (NDEA; 20 μg/kg bw) in ethanol (1ml; 5% v/v) led to a five-fold increase in *N*7 ethylation of guanine in oesophageal DNA over that seen with a similar dose of NDEA alone (Swann *et al.*, 1984; see also figure 10 in par. 4.1).

After ethanol intake, blood xylene levels in volunteers exposed to xylene by inhalation were increased 1.5-2.0 fold, while urinary excretion of methyl hippuric acid, a xylene metabolite, declined by about 50% (Riihimäki *et al.*, 1982). During exposure to toluene by inhalation (3.2 mmol/m^3) for 4.5 h, moderate doses of ethanol (15 mmol/kg body weight) given orally to volunteers almost doubled the maximum toluene concentration in blood and decreased the blood clearance of toluene by approximately 44% (Wallén *et al.*, 1984). Trichloroethylene concentrations in plasma increased twofold, and decreased urinary excretion of a major metabolite of trichloroethylene -trichloroethanol- was observed when ethanol was ingested immediately prior to exposure to trichloroethylene by inhalation (Müller *et al.*, 1975). Drinking of alcoholic beverages inhibits liver metabolism of nitrosamines, such as N-nitrosodimethylamine and N-nitrosodiethylamine. As a result, nitrosamines are excreted in urine of beer drinkers and volunteers given amines and ethanol (Eisenbrand *et al.*, 1981; Spiegelhader & Preussmann, 1985).

5. OUTLINE OF THE THESIS

Consumption of alcohol and tobacco smoking belong to the most common joint exposures in humans. A more than additional cancer risk due to this exposure has been observed in several organs (*Chapter I*). The purpose of the present study was to obtain insight in possible sites and levels of interaction. As stated in *Chapter I*, paragraph 4, interaction can take place at the exposition phase, at the toxicokinetic phase and at the toxicodynamic phase. Ethanol and B(*a*)P were chosen to represent exposure to alcoholic beverages and PAHs (major constituents of tobacco smoke, Bos & Henderson, 1984) respectively. In our studies possible interactions were investigated on the toxicokinetic level (metabolism and excretion) and on the toxicodynamic level (effect at target organ) (Table 3).

We started with the design of a method of ethanol administration to rats via the drinking water (*Chapter II*). A dosage procedure is developed that corresponds to 'moderate' drinking in humans. Moderate drinking is defined as long-term consumption of about 20% of the daily caloric intake as ethanol.

In an *in vivo* study, benzo(*a*)pyrene [B(*a*)P] metabolites excreted after oral and intraperitoneal administration were compared to detect differences between these routes of administration and to study the influence of ethanol-pretreatment. B(*a*)P-metabolites excreted in male and female rats were compared to detect sex differences and the inluence of ethanol-pretreatment (*Chapter III*).

Data on the effects of long-term ethanol treatment on the microsomal biotransformation of B(*a*)P are presented in *Chapter IV*. Microsomes of lung, liver and small intestine from male and female rats were used. In this way a comparison could be made of possible sex-related differences in B(*a*)P biotransformation in the three organs. To determine which isoenzyme of P450 was most important in B(*a*)P biotransformation, liver microsomes were incubated with monoclonal antibodies to the P450 isoenzymes 1A1\2, 2E1, 2B1\2 and 2C11\6 respectively, measuring the amount of inhibition of B(*a*)P metabolite formation.

The influence of long-term ethanol treatment on biotransformation capacity is monitored by measuring the metabolism of several substrates that are indicative for isoenzymes of P450. Ethoxyresorufin O-deethylase is measured as an indicator enzyme for the activity of P4501A1. The increase in aniline hydroxylation is used as a marker for ethanol-dependent increases in P4502E1. Pentoxyresorufin O-deethylase activity is used as a marker for P4502B1/P4502B2 isoenzymes. Testosteron metabolism is correlated with the sex specific isoenzymes. Besides P450 biotransformation activities, glutathion S-transferase activity and subunit composition were studied because of the important role this enzyme plays in the detoxification of reactive B(*a*)P metabolites. Because epoxide hydrolase plays an important role in detoxification and toxification of B(*a*)P, this

enzyme was also monitored. Again male and female rats were used to observe possible sex differences (*Chapter V*).

In *Chapter VI* and *Chapter VII* DNA adduct measurement in *in vivo* studies is described. DNA adducts may play an important role in the initiation of B(*a*)P-induced cancer. We choose them to measure the influence of ethanol-pretreatment on the toxicodynamic phase of B(*a*)P. In one study, the level of DNA adducts in liver and small intestine of ethanol-pretreated and non-pretreated rats was measured at various time intervals after a single oral dose of B(*a*)P In another study B(*a*)P was applied several times, combined with ethanol treatment DNA adducts were analyzed in oesophagus and forestomach, being target organs for B(*a*)P/ethanol carcinogenicity and in liver and small intestine as the major metabolizing organs

Table 3 sums up the sites of ethanol and B(*a*)P interaction and the measurements described in our study.

absorption distribution elimination	phenolic and diolic metabolites in urine and feces
metabolism	microsomes of liver, lung and small intestine
effect	DNA adducts in liver, lung and small intestine

Table 3 Sites of ethanol and B(*a*)P interaction investigated in our studies All assays were performed on (samples from) male and female rats chronically treated with ethanol, orally or intraperitoneally treated with B(*a*)P

6. REFERENCES

Aaseth J, Smith-Kielland A, Thomassen Y Selenium, alcohol and liver diseases Ann Clin Res 1986,18 43-7

Achord JL Nutrition, alcohol, and the liver Am J Gastroenterol 1988,83 244-8

Agarwal DP, Goedde HW Ethanol oxidation, ethnic variations in metabolism and response In Kalow-W, ed Ethnic Differences in Reactions to Drugs and Xenobiotics New York Alan R Liss, 1986 (Progress in Clinical and Biological Research Series, Vol 214)

Alderman J Partial characterization of hepatic acetaldehyde dehydrogenase from the baboon Prog Clin Biol Res 1982,114 77-89

Aluminium Association Inc Mortality of aluminium workers final report New York Aluminium Association Inc , 1977

Ames BN, Magaw R, Gold LS Ranking possible carcinogenic hazards Science 1987,236 271-80

Anderson P What is a safe level of alcohol consumption? Stress Medicine 1986,2 99-101

Anonymous Encyclopaedia Britannica Chicago William Benton, 1966

Anonymous Immunological abnormalities in alcoholic liver disease Lancet 1983,1 605-6

Anonymous Defeating the breathalyser Br Med J 1972,4 744-5

Austin H, Delzell E, Grufferman S, Levine R, Morrison AS, Stolley PD & Cole P A case-control study of hepatocellular carcinoma and the hepatitis B virus, cigarette smoking, and alcohol consumption Cancer Res 1986,46 962-6

Badger GM Mode of formation of carcinogens in human environment In Badger-GM, ed The chemical Basis of Carcinogenic Activity Springfield Charles C Thomas, 1962

Baird WM, Harvey RG & Brookes P Comparison of the cellular DNA-bound products of benzo(*a*)pyrene with the products formed by reaction of benzo(*a*)pyrene 4,5-oxide with DNA Cancer Res 1975,35 54-7

Baird SM, Beattie GM, Lennon RA, Lipsick JS, Jensen FC, Kaplan NO Induction of lymphoma in antigenically stimulated athymic mice Cancer Res 1982,42 198-206

Baraona E, Julkunen R, Tannenbaum L & Lieber CS Role of intestinal bacterial overgrowth in ethanol production and metabolism in rats Gastroenterology 1986,90 103-10

Baraona E, DiPadova C & Lieber CS Transport of acetaldehyde in red blood cells from liver to other tissues (Abstract No 405) Hepatology 1985,5 1048

Barry RE Role of acetaldehyde in the pathogenesis of alcoholic liver disease Br J Addict 1988,83 1381-6

Beck IT, Dinda PK Acute exposure of small intestine to ethanol, effects on morphology and function Dig Dis Sci 1981,26 817-38

Bell J Paraffin epithelioma of the scrotum Edinburgh Med J 1876, 22 135-7

Berggren SM, Goldberg L The absorption of ethyl alcohol from the gastro-intestinal tract as a diffusion process Acta Physiol Scand 1940,1 246-70

Best CH, Hartroft WS, Lucas CC, et al Liver damage produced by feeding alcohol or sugar and its prevention by choline Br Med J 1949,2 1001-6

Bird RP, Schneider R, Stamp D, Bruce WR Carcinogenesis 1986,7 1657

Bjorseth A & Becher G PAH in work atmospheres occurrence and determination Boca Raton CRC Press, 1986

Black SD & Coon MJ P-450 cytochromes structure and function Adv Enzymol Relat Areas Mol Biol 1987,60 35-87

Bloch B & Dreyfuss W Schweiz med Wschr 1921,51 1033-7

Blumer M Polycyclic aromatic compounds in nature Sci Am 1976,234 34

Bock RG & Dao TL Factors affecting the polynuclear hydrocarbon level in rat mammary glands Cancer Res 1961,21 1024

Boland B, Roizen R Sales slips and survey responses new data on the reliability of survey consumption measures Drinking Drug Pract Surv 1973,8 5-10

Borneff J & Kunte H Carcinogenic substances in water and soil 25 A routine method for the determination of polynuclear aromatic hydrocarbons in water Arch Hyg Bakt 1969,153 220-9

Bos JL Ras oncogenes in human cancer a review Cancer Res 1989,49 4682-9

Bos RP & Henderson P Th Genotoxic risk of passive smoking Rev Environ Health 1984,4(2) 161-78

Bosron WF, Li T-K, Valle BL New molecular forms of human liver alcohol dehydrogenase isolation and characterization of ADH Indianapolis Proc natl Acad Sci USA 1980,77 5784-8

Boyer TD The glutathione S-transferases an update Hepatology 1989,9 486-96

Buset M, Lipkin M, Winawer S, Swaroop S, Friedman E, Cancer Res 1986,46 5426

Butlin HT Three lectures on cancer of the scrotum in chimney sweepers and others Br med J 1892, 2 1341, 3 1, 3 66-71

Caballeria J, Baraona E, Rodamilans M, Lieber CS Effects of cimetidine on gastric alcohol dehydrogenase activity and blood ethanol levels Gastroenterology 1989,96 388-92

Caenegem RC et al Grote Winkler Prins Encyclopedie Amsterdam/Brussels Elsevier Argus, 1980 491

Camus A-M, Aitio A, Sabadie N, Wahrendorf J & Bartsch H Metabolism and urinary excretion of mutagenic metablites of benzo(*a*)pyrene in C57 and DBA mice strains Carcinogenesis 1984,5 35

Castonguay A, Rivenson A, Trushin N, Reinhardt J, Spathopoulos S, Weiss CJ, Reiss B & Hecht SS Effects of chronic ethanol consumption on the metabolism and carcinogenicity of N'-nitrosonornicotine in F344 rats Cancer Res 1984,44 2285-90

Centraal Bureau voor de Statistiek Compendium Gezondheidsstatistiek Nederland 1979 Ministerie van volksgezondheid en Milieuhygiene, Staatsuitgeverij 1980, 's-Gravenhage

Chouroulinkov I, Gentil A & Guérin Bulletin du Cancer 1967,54 67-

Clemmesen J Cancer and Occupation in Denmark, 1935-1939 Copenhagen Nyt Nordisk Forlag, 1941

Coles B & Ketterer B The role of glutathione and glutathione transferases in chemical carcinogenesis Crit Rev Biochem Mol Biol 1990,25 47-70

Committee on Biologic Effects of Atmospheric Pollutants, Particulate Polycyclic Organic Matter Washington Nat Academy of Sciences, 1972

Committee on Diet, Nutrition and Cancer, Assembly of Life Sciences, National Research Council Diet, Nutrition and Cancer Washington National Academy Press, 1982

Cook P Cancer of the oesophagus in Africa a summary and evaluation of the evidence for the frequency of occurrence, and a preliminary indication of the possible association with the consumption of alcoholic drinks made from maize Br J Cancer 1971,25 853-80

Coon MJ, Ding X, Pernecky SJ & Vaz ADN Cytochrome P450 progress and predictions FASEB J 1992,6 669-73

Daudel P, Duquesne M, Vigny P, Grover PL & Sims P Fluorescence spectral evidence that benzo(*a*)pyrene-DNA products in mouse skin arise from diol-epoxides FEBS Let 1975,57 250-3

Daynes RA, Harris CC, Connor RJ, Eichwald EJ Skin cancer development in mice exposed chronically to immunosuppressive agents J Natl Cancer Inst 1979,62 1075-81

DeCarli LM, Lieber CS Fatty liver in the rat after prolonged intake of ethanol with nutritionally adequate new liquid diet J Nutr 1967,91 331-336

Deutsch-Wenzel RP, Brune H, Grimmer G, Dettbarn G & Misfeld J Experimental studies in rat lungs on the carcinogenicity and dose-response relationships of eight frequently occurring environmental polycyclic aromatic hydrocarbons J Natl Cancer Inst 1983,71 539-44

Dicker E, Cederbaum AI Increased oxygen radical-dependent inactivation of metabolic enzymes by liver microsomes after chronic ethanol consumption FASEB J 1988,2 2901-6

Ding X, Koop DR, Crump BL & Coon MJ Immunochemical identification of cytochrome P450 isozyme 3a ($P450_{ALC}$) in rabbit nasal and kidney microsomes and evidence for differential induction by alcohol Mol Pharmacol 1986,30 370-8

Dipple A, Moschel RC & Bigger CAH Polynuclear aromatic hydrocarbons In Searle-CE, ed Chemical Carcinogens Vol I Second ed Washington American Chemical Society 1984 41-163

Dipple A Polynuclear aromatic carcinogens In Searle-CE, ed Chemical Carcinogens Washington American Chemical Society, 1976, (ACS Monogr 173)

Doll R, Vessey MP, Beasley RWR, Buckley AR, Rear EC, Fisher REW, Gammon EJ, Gunn W, Hughes GO, Lee K, Norman-Smith G Mortality of gas workers Final report of a prospective study Br J Ind Med 1972,29 394-406

Doll R & Peto R The causes of cancer Oxford Oxford Univ Press, 1981

Dutta SK, Dukehart M, Narang A, Latham PS Functional and structural changes in parotid glands of alcoholic cirrhotic patients Gastoenterology 1989,96 510-8

de Duve C & Boudhuin P Peroxisomes (microbodies and related particles) Physiol Rev 1966,46 323-57

Eddleston ALWF, Vento S Relevance of immune mediated mechanisms in progressive alcoholic liver injury In Thomson-AD, ed Mechanisms of alcoholic liver damage Oxford Pergaman Press, 1988

Eisenbrand G, Spiegelhalder B & Preussmann R Analysis of human biological specimens for nitrosamine contents In Bruce-WR, Correa-P, Lipkin-M, Tannenbaum-SR & Wilkins-TD, eds Gastrointestinal Cancer Endogenous Factors Cold Spring Harbor CSH Press, 1981 275-83, (Banbury Report 7)

Elmer GI, Meisch RA, Goldberg SR and George FR. Fixed-ratio schedules of oral ethanol self-administration in inbred mouse strains Psychopharmacology 1988,96 431-36

Environmental Protection Agency Carcinogen assessment of coke oven emission Final Report Washington US Environmental Protection Agency, Office of Health and Environmental Assessment, 1984, (EPA-600/6-82-003F)

Environmental Protection Agency (EPA) EPA-method 610 Washington US Department of Education, Health and Welfare, 1979

Eriksson K In Eriksson-K, Sinclair-JD, Kiianmaa-K, eds Animal Models in Alcohol Research London Academic Press, 1980

Eriksson K Genetic selection for voluntary alcohol consumption in the albino rat Science 1968,159 739-41

French SW Biochemical basis for alcohol-induced liver injury Clin Biochem 1989,22 41-9

Frezza M, di Padova C, Pozzato G, Terpin M, Baraona E, Lieber Cs High blood alcohol levels in women the role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism N Engl J Med 1990,322 95-9

Gabrial GN, Schrager TF & Newberne PM Zinc deficiency, alcohol and a retinoid association with esophageal cancer in rats J natl Cancer Inst 1982,68 785-9

Garro AJ, Espina N, Farinati F, Lieber CS Ethanol and the repair of deoxyribonucleic acid (DNA) Alchol Health Res World 1986,10(3) 26-7

Garro AJ & Lieber CS Alcohol and cancer Ann Rev Pharmacol Toxicol 1990,30 219-49

Gavaler JS Sex-related differences in ethanol-induced liver disease artificial or real? Alcoholism Clin Exp Res 1982,6 186-96

Gelboin HV Benzo[a]pyrene metabolism, activation, and carcinogenesis role and regulation Anonymus Alcohol and health Lancet 1987,I 876

Gezondheidsraad Polycyclische aromatische koolwaterstoffen (PAK) in de buitenlucht Advieswaarden voor de kwaliteit van de buitenlucht Den Haag Gezondheidsraad, 1984, (publikatienr 1984/40)

Gibbs GW Mortality of aluminium reduction plant workers, 1950 through 1977 J Occup Med 1985,27 761-70

Gibbs GW & Horowitz I Lung cancer mortality in aluminium reduction plant workers J Occup Med 1979,21 347-53

Gibel W, Lohs K, Wildner GP Experimentelle Untersuchungen zur karzinogenen Wirkung von Lösungsmitteln am Beispiel van Propanol-1,2-Methylpropanol und 3-Methylbutanol-1 Arch Geschwulstforsch 1975 45,19-24

Gibel W Experimental studies on syncarcinogenesis in oesophageal carcinoma (Ger) Arch Geschwulstforsch 1967,30 181-9

Glen I, Skinner F, Glen E, MacDonell L The role of essential fatty acids in alcohol dependence and tissue damage Alcoholism Clin Exp Res 1987,11 37-41

Goedde HW, Agarwas DP Aldehyde dehydrogenase polymorphism" molecular basis and phenotypic relationship to alcohol sensitivity In Lindros-KO, Ylikhari-R, Kiianmaa-K, eds Advances in biomedical alcohol research 3rd Congress of the International Society for Biomedical Research on Alcoholism, Helsinki, Finland, 8-13 June 1986 Oxford Pergamon Press, 1987, 47-54

Gold LS, Sawyer CB, Magaw R, Backman GM, de Veciana M, Levinson R, Hooper NK, Havender WR, Bernstein L, Peto R, Pike ML & Ames BN Environ Health Persp 1984,58 9-

Graham S, Dayal H, Rohrer T, Swanson M, Sultz H, Shedd D & Fischman S Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer J natl Cancer Inst 1977,59 1611-18

Grasso P Carcinogens in food In Searle-CE, ed Chemical Carcinogens, Vol II Washington American Chemical Society, 1984 1203-39

Griciute L, Castegnaro M & Bereziat JD Influence of ethyl alcohol on the carcinogenic activity of N-nitrosodi-n-propylamine In Bartsch-H, Castegnaro-M, O'Neill-IK & Okade-M, eds N-Nitroso Compounds Occurrence and Biological Effects Lyon IARC, 1982 643-8, (IARC Scientific Publications No 41)

Griciute L Influence of ethyl alcohol on experimental carcinogenesis In Proceedings of the 5th Conference of the Oncologists of Estonian SSR, Latvian SSR and Lithuanian SSR, 26-28 October, Tallin, USSR, 1981 170

Griciute L, Castegnaro M & Bereziat JC Influence of ethyl alcohol on carcinogenesis induced by volatile N-nitrosamines detected in alcoholic beverages In Bartsch-H, O'Neill-IK & Schulte-Hermann-R, eds Relevance of N-Nitroso Compounds to Human Cancer Exposures and Mechanisms Lyon IARC, 1987 264-5 (IARC Scientific Publications No 84)

Grimmer-G, ed Environmental Carcinogens Polycyclic Aromatic Hydrocarbons Boca Raton CRC Press, 1983

Grover PL, Hewer A, Pal K & Sims P The involvement of a diol-epoxide in the metabolic activation of benzo(*a*)pyrene in human bronchial mucosa and in mouse skin Int J Cancer 1976,18 1-6

Grunnet N Oxidation of acetaldehyde by rat-liver mitochondria in relation to ethanol oxidation and the transport of reducing equivalents across the mitochondrial membrane Eur J Biochem 1973,35 236-43

Guengerich FP, Kim D-H & Iwasaki M Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects Chem Res Toxicol 1991,4 168-79

Guengerich FP, Roles of cytochrome P450 enzymes in chemical carcinogenesis and cancer chemotherapy Cancer Res 1988,48 2946-54

Guengerich FP Reactions and significance of cytochrome P-450 enzymes J Biol Chem 1991,266 10019-22

Haggard HW, Greenberg LA, Lolli G The absorption of alcohol with special reference to its influence on the concentration of alcohol appearing in the blood Q J Stud Alcohol 1941,1 684-726

Hall M & Grover PL Polycyclic aromatic hydrocarbons metabolism, activation, tumour initiation In Cooper-CS and Grover-PL, eds Chemical Carcinogenesis and Mutagenesis, Vol I New York Raven Press, 1990 327-72

Hamilton SR, Hyland J, McAvinchey D, Chaudhry Y, Hartka L, Kim HT, Cichon P, Floyd J, Turjman N, Kessie G, Nair PP & Dick J Effects of chronic dietary beer and ethanol consumption on experimental colonic carcinogenesis by azoxymethane in rats Cancer Res 1987a;47:1551-9.

Hamilton SR, Sohn OS & Fiala ES Effects of timing and quantity of chronic dietary ethanol consumption on azoxymethane-induced colonic carcinogenesis and azoxymethane metabolism in Fischer 344 rats Cancer Res 1987b,47 4305-11

Hammond EC, Selikoff IJ, Lawther PL & Seidman H Inhalation of benzo(*a*)pyrene and cancer in man Ann NY Acad Sc 1976,271.116-24.

Harada S, Agarwal DP, Goedde HW. Aldehyde dehydrogenase deficiency as cause of facial fluching reaction to alcohol in Japanese. Lancet 1981,ii 982

Harger RN, Hulpieu HR, Lamb EB The speed with which various parts of the body reach equilibrium in the storage of ethyl alcohol J biol Chem 1937,120 689-704.

Hasamura Y & Takeuchi J Wechselbeziehungen zwischen Alkoholkonsum und Arzneimittelstoffwechsel in der Leber. Leber Magen Darm 1978,8 286-90.

Hawkins RD & Kalant H The metabolism of ethanol and its metabolic effects Pharmacol Rev 1972,24 67-157.

Heidelberger C & Weiss SM. The distribution of radioactivity in mice folowing administration of 3,4-benzo-pyrene-5-C^{14} and 1,2,5,6-dibenzanthracene-9,10-C^{14} Cancer Res 1951,11 885.

Hemminki K Environmental carcinogens In: Cooper-CS & Grover-PL, eds Chemical Carcinogenis and Mutagenesis, Vol I New York. Raven Press, 1990;33-62

Henderson BE et al, Cancer Res 1982;42.3232

Hieger I Spectra of cancer-producing tars and oils and of related substances Biochem J 1930,24 505-11.

Hieger K The isolation of a cancer producing hydrocarbon from coals tar J Chem Soc 1933,395

Hinds MW, Thomas DB & O'Reilly HP Asbestos, dental X-rays, tobacco and alcohol in the epidemiology of laryngeal cancer Cancer 1979;44.1114-20.

Hirayama T. A large-scale cohort study on the relationship between diet and selected cancers of digestive organs. In. Bruce-WR, Correa-P, Lipkin-M, Tannenbaum-SR & Wilkins-TD, eds. Castrointestinal Cancer Endogenous Factors Cold Spring Harbor CSH Press, 1981:409-429; (Banbury Report 7)

Hoffmann D & Hecht SS Advances in tobacco carcinogenesis In: Cooper-CS & Grover-PL, eds Chemical Carcinogenesis and Mutagenesis, Vol I New York: Raven Press, 1990, 63-102

Hogstedt C, Andersson K, Frenning B, Gustavsson A. A cohort on mortality among long time employed Swedish chimney sweeps Scand J Work Environ Health Suppl 1981;1:72-8.

Holden C. Probing the complex genetics of alcoholism. Science 1991;251·163-4

Holford NHG Clinical pharmacokinetics of ethanol Clin Pharmacokinet 1987,13:273-92.

Hopkins GJ & Carroll KK. J environ pathol toxicol oncol 1985;5:279.

Horie A, Kohchi S & Kuratsune. GANN 1965;56:429-

Horrobin DF. A biochemical basis for alcoholism and alcohol-induced damage including the fetal alcohol syndrome and cirrhosis: interference with essential fatty acid and prostaglandin metabolism. Med Hypotheses 1980;6:929-42

Horrobin DF. Essential fatty acids, prostaglandins, and alcoholism: an overview Alcoholism Clin Exp Res 1987;11:2-9.

Howarth AE & Pihl E. High-fat diet promotes and causes distal shift of experimental rat colonic cancer -beer and alcohol do not. Nutr Cancer 1985,6;229-35.

IARC. Polynuclear Aromatic Compounds: Part 1, Chemical, Environmental and Experimental Data Lyon: IARC, 1983; (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 32)

IARC Polynuclear Aromatic Compounds Part 3, Industrial exposures in aluminium production, coal gasification, coke production, and iron and steel founding Lyon IARC, 1983, (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 34)

IARC Overall Evaluations of Carcinogenicity An Updating of IARC Monographs Volumes 1 to 42 Lyon IARC, 1987, (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Suppl 7)

IARC Tobacco Smoking Lyon 1986, (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 38)

IARC Allyl Compounds, Aldehydes, Epoxides and Peroxides Lyon 1985, (Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 36)

IARC Alcohol drinking Lyon IARC, 1988, (IARC monographs on the evaluation of carcinogenic risks to humans, Vol 44)

Ijiri I Studies on the relationship between the concentrations of blood acetaldehyde and urinary catecholamine and the symptoms after drinking alcohol (Jpn) Jpn J Stud Alcohol 1974,9 35-59

Ingelman-Sundberg M, Ekstrom G, Tindberg N Lipid peroxidation dependent on ethanol-inducible cytochrome P-450 from rat liver In Nordman-R, Ribiere-C, Rouach-H, eds Alcohol toxicity and free radical mechanisms Proc 1st Congr ESBRA Paris, 18-19 September 1987 Oxford Pergamon Press, 1988 43-8, (Advances in the biosciences vol 71)

Inoue K, Aiba N, Nambu S, Sasaki H Influence of ethanol on hepatocarcinogenesis, site specific demethylation of c-myc oncogen in hepatocellular carcinoma (HCC) of heavy drinkers In Kuriyama-K, Takada-A, Ishii-H Biomedical and social aspects of alcohol and alcoholism Amsterdam Excerpta Medica, 1988 817-22

Ioannides C & Parke DV The cytochrome P-448 A unique family of enzymes involved in chemical toxicity and carcinogenesis Biochem Pharmacol 1987,36 4197-207

Ioannides C & Parke DV The cytochrome P450 1 gene family of microsomal hemoproteins and their role in the metabolic activation of chemicals Drug Metab Rev 1990,22 1-85

Isen OA, Lieber CS & Gottlieb LS The ultrastructure of fatty liver induced by prolonged ethanol ingestion Am J Pathol 1966,48 535-55

Israel Y, et al Hypermetabolic state, oxygen availability, and alcohol-induced liver damage In Majchrowicz-E, Noble-EP, eds Biochemistry and pharmacology of ethanol, Vol 1 New York Plenum Press, 1979 433-44

Israel Y, et al Studies on metabolic tolerance to alcohol, hepatomegaly and alcoholic liver disease Drug Alcohol Depend 1979,4 109-18

Isselbacher KJ Metabolic and hepatic effects of alcohol N Engl J Med 1977,296 612-6

Jacobsen E The metabolism of ethyl alcohol Pharmacol Rev 1952,4 107-35

Jacobson EA, Newmark HL, Bright-See E, McKeown-Eyssen G, Bruce WR, Nutr Rep Int 1984,30 1049

Jakoby WB, Bend JR & Caldwell J, eds Metabolic Basis of Detoxication Metabolism of Functional Groups New York Academic Press, 1985

Johnson PJ, Krasner N, Eddleston ALWF, Williams R Hepatocellular carcinoma in Great Brittain influence of age, sex, HBsAg status and aetiology of underlying cirrhosis Gut 1978,19 1022-26

Johnson, H The World Atlas of Wine, 3rd ed , London, Mitchell Beazley International, 1985

Joly J-G, Ishii H, Teschke R, Hasumura Y & Lieber CS Effect of chronic ethanol feeding on the activities and submicrosomal distribution of reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase and the demethylases for aminopyrine and ethylmorphine Biochem Pharmacol 1973,22 1532-5

Jones MK, Jones BM Ethanol metabolism in women taking oral contraceptives Alcoholism Clin Exp Res 1984,8 24-8

Jones BM, Jones MK Women and alcohol intoxication, metabolism and the menstrual cycle In Schuckit-MA, Greenblatt-M, eds Alcoholism problems in women and children New York Grune and Stratton, 1976 103-36

Jongeneelen FJ Biological monitoring of occupational exposure to polycyclic aromatic hydrocarbons (Thesis) Nijmegen Scientific Publishers, 1987

Jongeneelen FJ, Leeuwen FE van, Oosterink S, Anzion RBM, Loop F van der, Bos RP & Veen HG van Ambient and biological monitoring of cokeoven workers determinants of the internal dose of polycyclic aromatic hydrocarbons Brit J Ind Med 1990,47 454-61

Joossens JV, Hill MJ, Geboers J, eds Diet and Human Carcinogenesis Amsterdam Elsevier, 1985

Kalant H Absorption, diffusion, distribution, and elimination of ethanol effects on biological membranes In Kissin-B, Begleiter-H, eds The biology of alcoholism I New York Plenum Press, 1971 1-62

Kawai M, Amamoto H, Harada K Epidemiologic study of occupational lung cancer Arch Environ Health 1967,14 859-64

Keane B, Leonard BE Rodent models of alcoholism A Review Alcohol & Alcoholism 1989,24(4) 299-309

Keilin D & Hartree EF Coupled oxidation of alcohol Proc R Soc med (London) 1936,119 141-59

Keller M, McCormick M, Efron V A dictionary of words about alcohol New Brunswick Rutgers Center of Alcohol Studies Publication Division, 1982

Keller AZ & Terris M The association of alcohol and tobacco with cancer of the mouth and pharynx Am J publ Health 1965,55 1578-85

Keller A Alcoholic beverages and carcinogenesis NY State J Med 1980 1245-52

Kennaway EL & Kennaway NM A further study of the incidence of cancer of the lung and larynx Br J Cancer 1947,1 260-298

Kennaway EL & Hieger I Carcinogenic substances and their fluorescence spectra Br Med J 1930,1044-1046

Kiessling KH The occurrence of acetaldehyde in various parts of the rat brain after alcohol injection, and its effect on the pyruvate oxidation Exp Cell Res 1962,27 367-8

Kipling MD Soots, tars and oils as causes of occupational cancer In Searle-CE, ed Chemical Carcinogens Washington American Chemical Society, 1977

Klinger W Alkohol und Arzneimittelbiotransformation Z Ges Inn Med 1981,36 543-7

Knecht KT, Bradford BU, Mason RP, Thurman RG In vivo formation of a free radical metabolite of ethanol Mol Pharmacol 1990,38 26-30

Knudsen I, ed Genetic Toxicology of the Diet New York Liss, 1986

Kobayashi N, Hoffmann D & Wynder EL A study of tobacco carcinogenesis XII Epithelial changes in the upper respiratory tracts of Syrian golden hamsters by cigarette smoke J Natl Cancer Inst 1974,53 1085-9

Koga S, Hirayama C Disturbed release of lipoprotein from ethanol-induced fatty liver Experientia 1968,24 438-9

Konishi N, Kitahori Y, Shimoyama T, Takahashi M & Hiasa Y Effects of sodium chloride and alcohol on experimental esophageal carcinogenesis induced by N-nitrosopiperidine in rats Jpn J Cancer Res (Gann) 1986,77 446-51

Konstantinov VG, Alikin PF, Ustyuzhania ZV, Bessovnova AP, Gorina US Resinous substances and B(*a*)P in the air environment of some industrial buildings of the Dnjepr electrode plant Chem Abst 1979,91 43906

Koop DR, Morgan ET, Tarr GE and Coon MJ Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits J Biol Chem 1982 257 8472-80

Koop DR & Coon MJ Ethanol oxidation and toxicity role of alcohol P450 oxygenase Alcoholism Clin Exp Res 1986,10 44s-9s

Koop DR Oxidative and reductive metabolism by cytochrome P450 2E1 FASEB J 1992,6 724-30

Kopun M, Propping P The kinetics of ethanol absorption and elimination in twins and supplementary repetitive experiments in singleton subjects Eur J clin Pharmacol 1977,11 337-44

Kornet LMW Behavioural and neuropharmacological aspects of experimental alcohol addiction in Ghesus monkeys (Thesis) Utrecht State University, 1991

Konstantinov VG, Kyzmnikh AI Resinous substances and 3,4 benzpyrene in the aire of electrolysis shops of aluminium plants and their role in carcinogenesis Hyg sanit 1979,36 39-42

Kotin P, Falk HL & Busser R Distribution, retention, and elimination of C^{14}-3,4-benzpyrene after administration to mice and rats J Natl Cancer Inst 1959,23 541

Kramers PGN & van der Heijden CA Polycyclic Aromatic Hydrocarbons (PAH) Carcinogenicity data and risk extrapolations Toxicol Environ Chem 1988,16 341-51

Kuratsune M, Kohchi S, Horie A & Nishizumi M Test of alcoholic beverages and ethanol solutions for carcinogenicity and tumor-promoting activity Gann 1971,62 395-405

Lamy L Statistical clinical study of 134 cases of cancer of the oesophagus and cardia (Fr) Arch Mal Appar Digest 1910,4 451-75

Lang KF, Buffleb H & Zander M Pyrolysen von mehrkernigen aromatischen Kohlenwasserstoffen Erdol Kohle, 1963,16 944

Laser H Peroxidatic activity of catalase Biochem J 1955,61 122-7

Lasker JM, Ardies CM, Bloswick BP & Lieber CS Immunochemical evidence for an ethanol-inducible form of liver microsomal cytochrome P450 in rodents and primates Ped Proc 1986a,45 1665

Lasserre O, Flamant R, Lellouch J, Schwartz D Alcohol and cancer (Study of geographical pathology in French departments) Bull INSERM 1967,22 53-60

Leloir LF & Muñoz JM Ethyl alcohol metabolism in animal tissues Biochem J 1938,32 299-307

Lemon FR, Walden RT & Woods RW Cancer of the lung and mouth in Seventh-day Adventists Preliminary report on a population study Cancer 1964,17 486-97

Leo MA, Mak KM, Lieber CS Interaction of ethanol with vitamin A in liver and in tumor develpment Alcohol Health Res World 1986,10(3) 18-25

Liaw YF, Tai DI, Chu M Early detection of hepatocellular carcinoma in patients with chronic type B hepatitis Gastroenterology 1986,90 263-7

Li T-K, Bosron WF, Dafeldecker WP, Lange LG, Vallee BL Isolation of -Alcohol dehydrogenase of human liver is it a determinant of alcoholism? Proc natl Acad Sci USA 1977 74,4378-4381

Lieber CS Mechanism of ethanol induced hepatic injury Pharmacol Ther 1990,46 1-41

Lieber CS Ethanol metabolism and toxicity In Hodgson-E, Bend-JR & Philpot-RM, eds Reviews in Biochemical Toxicology, Vol 5 New York Elsevier 1983 267-311

Lieber CS Alcohol, protein nutrition, and liver injury Gastroenterology 1980 79,373-90

Lieber CS Liver adaptation and injury in alcoholism N Engl J Med 1973 288,356-62

Lieber CS & DeCarli LM Hepatic microsomal ethanol-oxidizing system in vitro characteristics and adaptive properties *in vivo* J biol Chem 1970,245 2505-12

Lieber CS New pathway of ethanol metabolism in the liver Gastroenterology 1970,59 930-7

Lieber CS & DeCarli LM Ethanol oxidation by hepatic microsomes adaptive increase after ethanol feeding Science 1968,162 917-8

Lieber CS, et al Differences in hepatic and metabolic changes after acute and chronic alcohol consumption Fedn Proc 1975,34 2060-74

Lieber CS Alcohol and the liver 1984 update Hepatology 1984,4 1243-60

Lieber CS, Seitz HK, Garro AJ, Worner TM Alcohol-related diseases and carcinogens Cancer Res 1979 39,2863-86

Lieber CS Medical disorders of alcoholism, pathogenesis and treatment Philadelphia Saunders, 1982, (Major problems in internal medicine 22)

Lieber CS Interaction of alcohol with other drugs and nutrients Drugs 1990a,40(S3) 23-44

Lieber CS, Garro AJ, Leo MA, Worner TM Mechanisms for the interrelationship between alcohol and cancer Alcohol Health Res World 1986 10(3),10-7

Lieber CS Interaction of ethanol with drugs, hepatotoxic agents, carcinogens and vitamins Alchol Alcoholism 1990b,25 157-76

Lieber CS, DeCarli LM The feeding of alcohol in liquid diets Two decades of applications and 1982 update Alcoholism Clin Exp Res 1982,6 523-31

Lieber CS, DeCarli LM, Sorrell MF Experimental Methods of Ethanol Administration Hepatology 1989,10(4) 501-11

Lieber CS Biochemical and molecular basis of alcohol-induced injury to liver and other tissues New England Journal of Medicine 319,1639-1650, 1988

Lindros KO Acetaldehyde its metabolism and role in the actions of alcohol Res Adv Alcohol Drug Probl 1978,4 111-76

Lindros KO Human blood acetaldehyde levels with improved methods, a clearer picture emerges Alcohol Clin Exp Res 1982,6 70-5

Lindros KO, Oshino N, Parrilla R & Williamson JR Characteristics of ethanol and acetaldehyde oxidation on flavin and pyridine nucleotide fluorescence changes in perfused rat liver J biol Chem 1974,249 7956-63

Linnoila M, Mattila MJ, Kitchell BS Drug interactions with alcohol Drugs 1979,18 299-311

Litvinov NN, Voronin VM & Kazatchkov VI Evaluation of carcinogenicity of nitrosodimethylamine used in combination with benzene, cadmium, boron or ethanol Vopr Onkol 1986,32 80-4

Litvinov NN, Voronin VM & Kazatchkov VI Studies of carcinogenesis modifying properties of aniline, carbon tetrachloride, benzene and ethanol Eksp Onkol 1986b,8 21-3

Lloyd WJ Long-term mortality study of steelworkers V Respiratory cancer in coke plant workers J Occup Med 1971,13 53-68

Loeb LA, Ernster VL, Warner KE, Abbots J & Laszlo J Smoking and lung cancer an overview Cancer Res 1984,44 5940-58

Lötterle J, Husslein EM, Bolt J, Wirtz PM Tageszeitliche Unterschiede der Alkoholresorption (Variations in alcohol absorption during the day) Blutalkohol 1989,26 369-75

Lu AYH & West CB Muluplicity of mammalian cytochrome P-450 Pharmacol Rev 1980,31 277-95

Lumeng L, Waller MB, McBride WJ and Li JK Different sensitivities to ethanol in alcohol preferring and non-preferring rats Pharmacol Biochem Behavior 1982,16 125-30

Lundquist F, Wolthers H The kinetics of alcohol elimination in man Acta Pharmacol Toxicol 1958,14 265-89

Majchrowitz E The role of blood in alcohol intoxication and addiction In Lisansky-Gomberg-E, Raskin-White-H, Carpenter-JA, eds Alcohol science and society revisited Ann Arbor University of Michigan Press, 1982 171-85

Makelä K, Room R, Single E, Sulkunen P, Walsh B, Bunce R, Cahannes M, Cameron T, Giesbrecht N, de Lint J, Mäkinen H, Morgan P, Mosher J, Moskalewicz J, Muller R, Österberg E, Wald I & Walsh D Alcohol, Society and the State 1 A Comparative Study of Alcohol Control Toronto Addiction Research Foundation, 1981

Mallach HJ Konzentrationsverlauf im Blut Pharmakokinetik In Mallach-HJ, Hartman-H, Schmidt-V Alkoholwirkung beim Menschen Pathophysiologie, Nachweis, Intoxication, Wechselwirkungen Stuttgart Thieme Verlag, 1987 13-34

Mannervik B the isoenzymes of glutathione transferase Adv Enzymol 1985,57 357-417

Mannervik B Glutathione Transferase In Benford-D, Bridhes-JW and Gibson-GG, eds Drug metabolism from molecules to man London Taylor and Francis Ltd, 1987 30-9

de Marini DM Genotoxicity of tobacco smoke and tobacco smoke condensate Mutat Res 1983,114 59-89

Marjanen LA Comparison of aldehyde dehydrogenases from cytosol and mitochondria of rat liver Biochim biophys Acta 1973,327 238-46

Martin E, Moll W, Schmidt P, Dettli L The pharmacokinetics of alcohol in human breath, venous and arterial blood after oral ingestion Eur J Clin Pharmacol 1984,26 619-26

Matsuda Y, Mori K, Hirohata T, Kuratsune M Carcinogenesis in the esophagus III Polycyclic aromatic hydrocarbons and phenols in whiskey Gann 1966 75,549-57.

Mazumdar S, Redmond C, Sollecito W, Sussman N An epidemiological study of exposures to coal tar pitch volatiles among coke oven workers J Air Pollut Control Assoc 1975,25 382-9

McCoy GD, Hecht SS, Katayama S & Wynder EL Differential effect of chronic ethanol consumption on the carcinogenicity of N-nitrosopyrrolidine and N'-nitrosonornicotine in male Syrian golden hamsters Cancer Res 1981,41 2849-54

McDonald J Moderate amounts of alcoholic beverages and clinical nutrition J Nutr Educ 1982,14 58-60

McGlashan ND Oesophagal cancer and alcoholic spirits in Central Africa Gut 1969,10 643-50

McGoy GD, Wynder EL Etiological and preventive implications in alcohol carcinogenesis Cancer Res 1979,39 2844-50

McKillop D & Case DE Mutagenicity, carcinogenicity and toxicity of ß-naphtoflavone, a potent inducer of P448 Biochem Pharmacol 1991,41 1-7

Mennes, WCM Species Differences in biotransformation (application of primary hepatocyte cultures derived from rat, hamster, monkey and man) (Thesis) Utrecht State university, 1992

Midanik L The validity of self-reported alcohol consumption and alcohol problems a literature review Br J Addict 1982,77 357-82

Miller EC & Miller JA Biochemical mechanisms of chemical carcinogenesis In Busch-HM, ed The Molecular Biology of Cancer New York Academic Press, 1973 377

Mills PR, Pennington TH, Kay P, MacSween RNM, Watkinson G Hepatitis B antibody in alcoholic cirrhosis J Clin Pathol 1979,32 778-82

Misulis SP & Garske A Induction of alcohol dehydrogenase in liver and gastro-intestinal tract Aust Ann Med 1969,18 227-31

Mitchell CE The metabolic fate of benzo(*a*)pyrenen in rats after inhalation Toxicology 1983,28 65

Mitchell CE Distribution and retention of benzo(*a*)pyrene in rats after inhalation Toxicol Lett 1982,11 35

Morel F, Beaune P, Ratanasavanag D, Flinois J-P, Guengerich FP & Guillouzo A Effects of various inducers on the expression of cytochromes P450 IIC8, 9, 10 and IIIA in cultured adult human hepatocytes Toxic in Vitro 1990,4 458-60

Moser J, ed Alcohol Policies in National Health and Development Planning Geneva WHO, (WHO Offset Publication No 89)

Mufti SI, Darban HR, Watson RR Alcohol, cancer and immunomodulation CRC Crit Rev Oncol/Hematol 1989,9 243-61

Muller G, Spassowski M & Henschler D Metabolism of trichloroethylene in man II Interaction of trichloroethylene and ethanol Arch Toxicol 1975,33 173-89

van der Naald WGH, Tamis W, van den Berg MMHE Polycyclische aromatische koolwaterstoffen in het Nederlandse milieu Leidschendam VROM-CCRX, 1988

Nakajima T, Elovaara E, Park SS, Gelboin HV, Hietanen E & Vainio H Monoclonal antibody directed characterization of benzene, ethoxyresorufin and pentoxyresorufin metabolism in rat liver microsomes Biochem Pharmacol 1990,40 1255-61

Neal J & Rigdon RH Texas Rep Biol Med 1967,25 553-

Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R & Waterman MR The P450 gene superfamily recommended nomenclature DNA,1987 6,1-11

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ The P450 superfamily update on new sequences, gene mapping, and recommended nomenclature DNA Cell Biol 1991,10 1-14

Nebert DW & Gonzalez FJ P-450 genes structure, evolution and regulation Ann Rev Biochem 1987,56 945-93

Nebert DW & Jones JE Regulation of the mammalian cytochrome P_1-450 (CYP1A1) gene Int J Biochem 1989,21 243-52

Nelson RL & Samelson SL Neither dietary ethanol nor beer augments experimental colon carcinogenesis in rats Dis Colon Rectum 1985,28 460-3

Newmark HL et al , Large Bowel Cancer In Mastromarino-AJ & Brattain-MG, eds Vol 3 in Cancer Research Monographs New York Praeger, 1985 102-30

NIOSH Coal tar pitch volatiles, method PCAM 217, Washington NIOSH, 1977

NIOSH Criteria for a Recommended Standard Occupational Exposure to Coal Tar Products Washington NIOSH, 1977, (Publ No 78-107)

Obe G, Ristow H Mutagenic, carcinogenic and teratogenic effects of alcohol Mutation Res 1979,65 229-59

Oesch F Microsomal epoxide hydrolase In Jakoby-WB, ed Enzymatic Basis of Detoxication Vol II London Academic Press, 1980, 277-90

Olsen H, Morland J Ethanol-induced increase in drug acetylation in man and isolated rat liver cells Br Med J 1978,ii 1260-62

Olson RE Absorption, metabolism and excretion of ethanol including effects on water balance and nutritional status In Gastineau-CF, Darby-WJ, Turner-TB, eds Fermented food beverages in nutrition New York Academic Press, 1979 197-212

Orme-Johnson WH & Ziegler DM Alcohol mixed function oxidase activity of mammalian liver microsomes Biochem biophys Res Commun 1965,21 78-82

Paget J Chimney-sweeper's cancer on the ear Lancet 1950,ii 265

Parilla R, Ohkawa K, Lindros KO, Zimmerman U-JP, Kobayashi K & Williamson JR Functional compartmentation of acetaldehyde oxidation in rat liver J biol Chem 1974,249 4926-33

Pawan GLS Metabolism of alcohol (ethanol) in man Proc Nutr Soc 1972,31 83-9

Pelkonen O & Sotaniemi EA Environmental factors of enzyme induction and inhibition Pharmac Ther 1987,33 115-20

Peng R, Tu YY & Yang CS The induction and competitive inhibition of a high affinity microsomal nitrosodimethylamine demethylase by ethanol Carcinogenesis 1982,3 1457-61

Pernanen K Validity of survey data on alcohol use In, Gibbins-RJ, Israel-Y, Kalant-H, Popham-RE, Schmidt-W, Smart-RG, eds Research Advances in Alcohol and Drug Problems Vol 1 New York John Wiley and Sons, 1974 355-74

Peto R & zur Hausen H, eds Viral etiology of cervical Cancer (Cold Spring Harbor Cold Spring Habor Lab, 1986 (Banbury Report 21)

Phillips RL, Garfinkel L, Kuzma JW, Beeson WL, Lotz T & Brin B Mortality among California Seventh-day Adventists for selected cancer sites J natl Cancer Inst 1980,65 1097-107

Phillips DH Fifty years of benzo(*a*)pyrene Nature 1983,303 468-72

Phillips DH Modern Methods of DNA adduct determination In Cooper-CS and Grover-PL, eds Chemical Carcinogenesis and Mutagenesis Vol I New York Raven Press, 1990 503-45

Picket CB, & Lu AYH Glutathione S-transferases gene structure, regulation, and biological function Ann Rev Biochem 1989,58 743-64

Pignatelli B, Castegnaro M, Walker EA Effects of gallic acid and ethanol on formation of nitroso-diethylamine Lyon International Agency for Research on Cancer, 1976 173-178 (IARC Science Publication 14)

Porter TD & Coon MJ Cytochrome P-450 multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms J Biol Chem 1991,266 13469-72

Pott P, Cancer scroti, in Chirurgical Observations, 1775 (Reprint) Natl Cancer Inst Monogr 1963,10 7

Pott F & Oberdorfer G Intake and distribution of PAH, in Environmental Carcinogens Polycyclic Aromatic Hydrocarbons In Grimmer-G, ed Boca Raton CRC Press, 1983 130

Pour FM, Reber HA & Stepan K Modification of pancreatic carcinogenesis in the hamster model XII Dose-related effect of ethanol J natl Cancer Inst 1983,71 1085-7

Projectgroep Alcohol Voorlichtings Plan van Iwaarden MJ, ed Feiten over alcohol Rijswijk WVC Centrale Directie Voorlichting, Documentatie en Bibliotheek

Racker E Aldehyde dehydrogenase, a diphosphopyridine nucleotide-linked enzyme J biol Chem 1949,177 883-92

Radike MJ, Stemmer KL & Bingham E Effect of ethanol on vinylchloride carcinogenesis Environ Health Perspect 1981,41 59-62

Raucy J, Fernandes P, Black M, Yang SL & Koop DR. Identification of a human liver cytochrome P450 exhibiting catalytic and imunochemical similarities to cytochrome P-450 exhibiting catalytic and immunochemical similarities to cytochrome P-450 3a of rabbit liver Biochem Pharmacol 1087,36 2921-6

Reddy MV, Gupta RC, Randerath E & Randerath K ^{32}P-Postlabeling test for covalent binding of chemicals *in vivo* application to a variety of aromatic carcinogens and methylation agents Carcinogenesis 1984,5 231-4

Redmond CK, Strobino BR & Cypess RH Cancer experience among coke by-product workers Ann NY Acad Sci 1976,271 102-15

Reed TE, Kalant H, Gibbins RJ, Khanna BM Alcohol and acetaldehyde metabolism in Caucasians, Chinese and Amerinds Can Med Assoc J 1976,115 851-5

Reinke LA, Moyer MJ & Notley KA Diminished rates of glucuronidation and sulfation in perfused rat liver after chronic ethanol administration Biochem Pharmacol 1986,35 439-47

Reinke LA, Lai EK, MacCay PB Administration of ethanol or acetaldehyde to rats results in the generation of free radicals in liver, heart, and other organs In Kuriyama-K, Takada-A, Ishii-H, eds Biomedical and social aspects of alcohol and alcoholism Proc 4th Congr ISBRA, Kyoto, Japan, 26 June-2 July 1988 Amsterdam Elsevier Science Publishers, 1988 663-6

Ricciardi BR. Hepatic ADH and AlDH isoenzymes in different racial groups and in chronic alcoholism Pharmacol Biochem Behav 1983 18,61-5

Rigdon RH & Neal J Texas Rep Biol Med 1966,24 195-

Riihimäki V, Savolainen K Pfäffli P, Pekari K, Sippel HW & Laine A Metabolic interaction between m-xylene and ethanol Arch Toxicol 1982,49 253-63

Room R. Measurements of drinking patterns in the general population and possible applications in studies of the role of alcohol in cancer Cancer Res 1979,39 2830-33

Rothman KJ & Keller A The effect of joint exposure to alcohol and tobacco on risk of cancer of the mouth and pharynx J chron Dis 1972,25 711-6

Rothman KJ The estimation of synergy or antagonism Am J Epidemiol 1976,103 506-11

Rubin E & Lieber CS Hepatic microsomal enzymes in man and rat induction and inhibition by ethanol Science 1968a,162 690-1

Rubin E, Gang H & Lieber CS Interaction of ethanol and pyrazole with hepatic microsomes Biochem Biophys Res Commun 1971,42 1-8

Rubin E, Hutterer F Ethanol increases hepatic smooth endoplasmatic reticulum and drug metabolizing enzymes Science 1968b,159 1469-70

Ryan DE & Levin W Purification and characterization of hepatic microsomal cytochrome P-450 Pharmacol & Ther 1990,45 153-239

Saffiotu U, Sunson SF, Keenan KP & McDowell EM Tumor enhancement factors and mechanisms in the hamster respiratory tract carcinogenesis model In Mass-MJ et al , eds Carcinogenesis, a Comprehensive Survey Vol 8 New York Raven Press, 1985 63-92

Sakabe H, Tsuchiya K, Takekura N, Nomura S, Koshi S, Takemoto K, Matsushita H, Matsuo Y Lung cancer among coke over workers Ind Health 1975,13 57-68

Salaspuro MP & Lindros K Metabolism and toxicity of acetaldehyde In Seitz-HK & Kommerell-B, eds Alcohol Related Diseases in Gastroenterology Berlin Springer 1985 106-23

Santodonato J, Howard P & Basu D J Environ Pathol Toxicol 1981,5 1-

Saracci R & Riboli E Passive smoking and lung caner current evidence and ongoing studies at the International Agency for Research on Cancer Mutat Res 1988,222 117-27

Schmahl D Investigations on esophageal carcinogenicity by methylphenylnitrosamine and ethyl alcohol in rats Cancer Lett 1976,1 215-18

Schmidt V Alkoholnachweis im Korper In Mallach-HJ, Hartmann-H, Schmidt-V, eds Alkoholwirkung beim Menschen Pathophysiologie, Nachweis, Intoxikation, Wechselwirkungen Stuttgart Georg Thieme, 1987 54-69

Schnellmann RG, Wiersma DA, Randall DJ, Smith TL & Sipes IG Hepatic mixed function oxygenase activity and glutathione S-transferase activity in mice following ethanol consumption and withdrawal Toxicology 1984,32 105-16

Schoemaker-Hol M A M , Sociaal drinken is asociaal Intermediair 1988,24(27) 47-51

Schwab GE & Hohnson EF Enzymology of rabbit cytochromes P-450 In Guengerich-FP, ed Mammalian Cytochromes P-450 Boca Raton CRC Press, 1987 55-105

van Schooten FJ, Polycyclic aromatic hydrocarbon-DNA adducts in mice and humans (Thesis) Amsterdam Cancer Institute Antoni van Leeuwenhoek, 1991

Seitz HK, Czygan P, Simanowski U, Waldherr R, Veith S, Raedsch R & Kommereel B Stimulation of chemically induced rectal carcinogenesis by chronic ethanol ingestion Alcohol Alcohol 1985,20 427-33

Seitz HK, Czygan P, Waldherr R, Veith S, Raedsch R, Kässmodel H & Kommerell B Enhancement of 1,2-dimethylhydrazine-induced rectal carcinogenesis following chronic ethanol consumption in the rat Gastroenterology 1984,86 886-9

Seitz HK, Bösche J, Czygan P, Veith S & Kommerell B Microsomal ethanol oxidation in the colonic mucosa of the rat Effect of chronic ethanol ingestion Naunyn-Schmiedeberg's Arch Pharmacol 1982,320 81-4

Seitz HK, Korsten MA & Lieber CS Ethanol oxidation by intestinal microsomes increased activity after chronic ethanol administration Life Sci 1979,25 1443-8

Seitz HK, Garro AJ, Lieber CS Effect of chronic ethanol ingestion on intestinal metabolism and mutagenicity of benzo(*a*)pyrene Biochem Biophys Res Commun 1978,85 1061-6

Sesardic D, Edwards RJ, Davies DS, Thomas PE, Levin W & Boobis AR High affinity phenacetin O-deethylase is catalysed specifically by cytochrome P450d (P450IA2) in the liver of the rat Biochem Pharmacol 1990,39 490-506

Sesardic D, Pasanen M, Pelkonen O & Boobis AR Differential expression and regulation of members of the cytochrome P450 1A gene subfamily in human tissues Carcinogenesis 1990a,1183-8

Shaper AG, Wannamethee G, Walker M Alcohol and mortality in British men explaining the U-shaped curve Lancet 1988,ii 1267-73

Silverstein M, Maizlish N, Park R, Mirer F Mortality among workers exposed to coal tar pitch volatiles and welding emissions an exercise in epidemiologia triage Am J Public Health 1985,75 1283-8

Sims P, Grover PL, Swaisland A, Pak K & Hewer A Metabolic activation of benzo(*a*)pyrene proceeds by a diol-epoxide Nature 1974,252 326-8

Singer B & Grunberger D Molecular biology of mutagens and carcinogens New York Plenum Press, 1983

Slooff w, Janus JA, Matthysen AJCM, Montizaan GK & Ros JPM, eds Integrated criteria document PAHs Bilthoven National Institute of Public Health and Environmental Protection, 1989, (Report number 758474007)

Smith M, Hopkinson DA, Harris H Studies on the subunit structure and molecular size of the human alcohol dehydrogenase isozymes determined by the different loci, ADH_1, ADH_2 and ADH_3 Ann Hum Genet 1973,36 401-14

Smith M, Hopkinson DA, Harris H Developmental changes and polymorphism in human alcohol dehydrogenase Ann Hum Genet 1971,34 251-71

Spector WS, ed Handbook of Biological Data Philadelphia Saunders, 1956

Spiegelhader B & Preussmann R. In vivo nitrosation of amidopyrine in humans use of 'ethanol effect' for biological monitoring of N-nitrosodimethylamine in urine Carcinogenesis 1985,6 545-8

Steward AR, Danna GA, Guzelian PS & Guengerich FP Changes in the concentration of seven forms of cytochrome P-450 in primary cultures of adult rat hepatocytes Mol Pharmacol 1985,27 125-32

Stewart RB and Grupp LA Conditioned place aversion mediated by selfadministered ethanol in the rat a consideration of blood ethanol levels Pharmacol Biochem & Behavior 1989,32 431-7

Stowers SJ, Maronpot RR, Reynolds SH and Anderson MW The role of oncogenes in chemical carcinogenesis Environ Health Perspect 1987,75 81-6

Sun JD, Wolff RK & Kanapilly GM Deposition, retention and biological fate of inhaled benzo(*a*)pyrene adsorbed onto ultrafine particles and as a pure aerosol Toxicol Appl Pharmacol 1981,65 231

Sundheimer DW & Brendel K Factors influencing sulfation in isolated rat hepatocytes Life Sci 1984,34 23-9

Swann PF Effect of etanol on nitrosamine metabolism and distribution implications for the role of nitrosamine in human cancer and for the influence of alcohol consumption on cancer incidence Lyon International Agency for Research on Cancer, 1984,501-12, (IARC Science Publications 57)

Sweeny DJ & Reinke LA Effect of ethanol feeding on hepatic microsomal UDP-glucuronyltransferase activity Biochem Pharmacol 1987,36 1381-3

Takahashi M, Hasegawa R, Furukawa F, Toyoda D, Sato H & Hayashi Y Effects of ethanol, potassium metabisulfite, formaldehyde and hydrogen peroxide on gastric carcinogenesis in rats after initiation with N-methyl-N'-nitro-N-nitrosoguanidine Jpn J Cancer Res (Gann) 1986,77 118-24

Teschke R, Minzlaff M, Oldiges H & Frenzel H Effect of chronic alcohol consumption on tumor incidence due to dimethylnitrosamine administration J Cancer Res clin Oncol 1983,106 58-64

Thijsse TH R & Huygen C Onderzoek naar de grootschalige achtergrondconcentraties van spoorelementen en -verbindingen in de Nederlandse buitenlucht (Investigations on the concentrations of trace-elements and -compounds in Dutch ambient air) Utrecht PEO Stichting Projekt Beheer Bureau Energie Onderzoek, 1985 (Report no R85/272)

Thomas PE, Bandiera S, Maines SL, Ryan DE & Levin W Regulation of cytochrome P450j, a high-affinity *N*-nitrosodimethylamine demethylase, in rat hepatic microsomes Biochemistry 1987,26 2280-9

Thyssen J, Althoff J, Kimmerle G & Mohr U Inhalation studies with benzo(*a*)pyrene in Syrian golden hamsters J Natl Cancer Inst 1981,66 575-7

Timms C, Schladt L, Robertson L, Rauch P, Schramm H & Oesch F The regulation of rat liver epoxide hydrolases in relation to that of other drug-metabolizing enzymes In Drug metabolism from molecules to man In Benford-D, Bridges-JW & Gibson-GG, Eds London Taylor and Francis, 1987 55-68

Tsutsui H Uber das künstlich erzeugte Canceroid bei der Maus, Gann 1918,12 17-21

Tsyrlov IB & Duzchak TG Interspecies features of hepatic cytochromes P450 IA1 and P450 IA2 in rodents Xenobiotica 1990,20 1163-70

Tsutsumi R, Leo MA, Cho-il Kim, Tsutsumi M, Lasker J, Lowe N, Lieber CS Interaction of ethanol with enflurane metabolism and toxicity role of P450IIE1 Alcohol Clin exp Res 1990,14 174-9

Tuyns AJ, Estève J, Raymond L, Berrino F, Benhamou E, Blanchet F, Boffetta P, Crosignani P, de Moral A, Lehmann W, Merletti F, Péquignot G, Riboli E, Sancho-Garnier H, Terracini B, Zubiri A & Zubiri Z Cancer of the larynx/hypopharynx, tobacco and alcohol Int J Cancer 1988 41,483-491

Tuyns AJ, Perquignot G & Abbatucci JS Oesophageal cancer and alcohol consumption importance of type of beverage Int J Cancer 1979a,23 443-7

Tuyns AJ, Pequignot G & Jensen OM Oesophageal cancer in Ille-et-Vilaine in relation to alcohol and tobacco consumption multiplicative risks (Fr) Bull Cancer 1977,64 45-60

Tuyns AJ, Estève J, Raymond L, Berrino F, Benhamou E, Blanchet F, Boffetta P, Crosignani P del Moral A, Lehmann W, Merletti F, Péquignot G, Riboli E, Sancho-Garnier H, Terracini, B, Zubiri A & Zubiri Z Cancer of the larynx/hypopharynx, tobacco and alchol Int J Cancer 1988,41 483-91

Tuyns AJ Cancer and alcoholic beverages In Gastinau-CF, Darby-WJ, Turner-TB, eds Fermented food beverages in nutrition New York Academic Press, 1979b 427-38

Tweedie JH, Reber HA, Pour P & Ponder DM Protective effect of ethanol on the development of pancreatic cancer surg Forum 1981,32 222-4

Ueng T-H, Friedman FK, Miller H, Park SS, Gelboin HV & Alvares AP Studies on ethanol-inducible cytochrome P450 in rabbit liver, lungs and kidneys Biochem Pharmacol 1987,36 2689-91

Umeno M, McBride OW, Yang CS, Gelboin HV & Golzalez FJ Human ehtanol-inducible P450IIE1 complete gene sequence, promoter, characterization, chromosome mapping, and cDNA-directed expression Biochemistry 1988,27 9006-13

Vaeck L van & van Cauwenberge K Characteristic parameters of particle size distributions of primary organic constituents of ambient aerosols Env Sci Techn 1985,19(8) 707

Vaessen HAM, van de Kamp CG & Jekel AA Evaluation of the results of intercomparison of methods for polycyclic aromatic hydrocarbons (PAH's) in food Bilthoven National Institute of Public Health and Environmental Protection (Report no 388700 002)

Vaessen HMG, Jekel AA & Wilbers AAMM Dietary intake of polycyclic aromatic hydrocarbons Tox and Environm Chem 1988,16 281-94

Verhoeve P & Compaan H Polycyclische aromatische koolwaterstoffen in het Nederlandse milieu Een oriënterende literatuurstudie TNO, 1980 (rapport CL 80/37, herziene uitgave)

Versluys JJ Cancer and occupation in the Netherlands Br J Cancer 1949,3 161-85

Vesell ES, Page JG, Pssananti GT Genetic and environmental factors affecting ethanol metabolism in man Clin Pharmacol Ther 1971,12 192-201

Von Volkmann R Beiträge zur Chirurgie Leipzig Breitkopf und Hartel, 1875

de Vos RH, van Dokkum W, Schouten A & de Jong-Berkhout P Polycyclic aromatic hydrocarbons in Dutch total diet samples (1984-1986) Fd Chem Toxic 1990,28 263-8

Vrij-Standhardt WG Alcohol metabolism In van de Heij-DG & Schaafsma-G, eds Biomedical and social aspects of alcohol use a review of the literature Wageningen Pudoc, 1991a

Vrij-Standhardt, WG Differences between alcoholic beverages In van der Heij-DG & Schaafsma-G, eds Biomedical and social aspects of alcohol use a review of the literature Wageningen Pudoc, 1991b

Wagner JG et al Elimination of alcohol from human blood J Pharm Sci 1976,65 152-4

Wällen M, Näslund PH & Nordqvist MB The effects of ethanol on the kinetics of toluene in man Toxicol appl Pharmacol 1984,76 414-9

Waller MB, McBride WJ, Gatto GJ, Lumeng L and Li T-K Intra-gastric self-infusion of ethanol by ethanol preferring and non-preferring lines of rats Science 1984,225 78-80

Wallgren H, Barry H. Actions of Alcohol, Vol. 1 Biochemical, Physiological and Psychological Aspects Amsterdam Elsevier, 1970.

von Wartburg JP, Schurch PM. Atypical human liver alcohol dehydrogenase Ann N Y Acad Sci 1968,151:936-46.

von Wartburg JP. Biochemische Auswirkungen des Alkoholismus Biblthca Nutr Dieta 1976,24·7-16

Waxman DJ. Interactions of hepatic cytochromes P450 with steroid hormones Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. Biochem Pharmacol 1988;37.71-84.

Weatherall DJ, Ladingham JGG & Warrell DA. Oxford Textbook of Medicine, Vol 1 Oxford: Oxford University Press, 1983.

Wehman HJ, Plantholt BA. Asbestos fibrils in beverages I. Gin Bull Envir Contam Toxicol 1974;11:267

Weinstein IB. The origins of human cancer: molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment. Twenty-seventh GHA Clowes memorial award lecture. Cancer Res 1988;48.4135-43.

Weinstein IB, Jeffrey AM, Jennette KW, Blobstein SH, Harvey RG, Harris CC, Autrup H, Kasai H & Nakanishi K. Benzo(*a*)pyrene diol epoxides as intermediates in nucleic acid binding *in vitro* and *in vivo*. Science 1976;193.592.

Westin S, Ström A, Gustafsson J-Å & Zaphiropoulos PG. Growth hormone regulation of the cytochrome P-450IIC subfamily in the rat. inductive, repressive, and transcriptional effects on P-450f (IIC7) and P-450$_{PB1}$ (IIC6) gene expression Mol Pharmacol 1990,38.634-43

Weston A, Grover PL & Sims P Metabolic activation of benzo(*a*)pyrene in human skin maintained in short-term organ culture Chem Biol Interact 1983,45·359-941.

WHO Guidelines for drinking water quality, Vol 1. Recommendations Geneva· WHO, 1984 130

WHO Working Group on Establishing Guidelines for Organic Carcinogenic Air Pollutants Polynucear Aromatic Hydrocarbons (PAH) Geneva: WHO, 1986.

WHO. Problems related to Alcohol Consumption Geneva WHO, 1980

WHO Polynuclear Aromatic Hydrocarbons Geneva· WHO, 1986

WHO World health statistics annual. Section A: global overview Alcohol consumption around 1977 and trends in beer consumption for selected regions 1960-1981 Geneva WHO, 1987.

Wickramasinghe SN, Barden G & Levy L The capacity of macrophages from different murine tissues to metabolise ethanol and generate an ethanol-dependent non-dialysable cytotoxic activity *in vitro* Alchol Alcohol. 1987;22.31-9.

te Wierik E, Moderate and excessive drinking, problem drinking and alcoholism. Definitions In van der Heij-DG & Schaafsma-G, eds. Biomedical and social aspects of alcohol use a review of the literature. Wageningen: Pudoc, 1991.

Wilkinson PK. Pharmacokinetics of ethanol: a review. Alcohol Clin Exp Res 1980,4:6-21

Willey JC, Grafstrom RC, Moser CE Jr Ozanne C, Sundqvist K & Harris CC Biochemical and morphological effects of cigarette smoke condensate and its fractions on normal human bronchial epithelial cells *in vitro*. Cancer Res 1987,47.2045-9.

Wortelboer HM Primary hepatocyte cultures as a model system for the determination of induction of biotransformation enzymes (Effects of glucosinolate hydrolysis products). (Thesis) Utrecht. State university of Utrecht, 1992.

Wynder EL, Lemon FR & Bross IJ. Cancer and coronary artery disease among Seventh-day Adventists. Cancer 1959;12:1016-28

Wynder EL, Bross IJ & Feldman RM. A study of etiological factors in cancer of the mouth. Cancer 1957;10:1300-23.

Wynder EL & Hoffmann D Tobacco. In. Schottenfeld-D and Fraumeni-JF, eds. Cancer Epidemiology and Prevention Philadelphia: WB Saunders, 1982. 277-92.

Yamagiwa K & Ichikawa K Über die kunstliche Erzeugung von Papillom, Verh Jpn Pathol Ges 1915,5 142-8

Yamamoto RS, Korzis J & Weisburger JH Chronic ethanol ingestion and the hepatocarcinogenicity of N-hydroxy-N-2-fluorenylacetamide Int J Cancer 1967,2 337-43

Yang SK, Mushtaq M & Chiu P-L Stereoselective metabolism and activations of polycyclic aromatic hydrocarbons In Harvey-RG, ed Polycyclic Hydrocarbons and Carcinogenesis Washington, American Chemical Society, 1984 19-34 (ACS symposium series no 283)

Yeh F-S et al, Cancer Res 1985,45 872

Yoshida A, Impraim CC, Huang I-Y Enzymatic and structural differences between usual and atypical human liver alcohol dehydrogenases J biol Chem 1981,256 12430-6

Yoshida A Differences in the isozymes involved in alchol metabolism between Caucasians and Orientals Isozymes Curr Top Biol Med Res 1983,8 245-61

Young M & Russell WT An Investigation into the Statistics of Cancer in Different Trades and Professions London Her Majesty's Stationery Office, 1926

Zagraninski RT, Kelsey JL & Walter SD Occupational risk factors for laryngeal carcinoma, Connecticut, 1975-1980 Am J Epidemiol 1986,124 67-76

Zaphiropoulos PG, Ström A, Robertson JA & gustafsson J-Å Structural and regulatory analysis of the male-specific rat liver cytochrome P-450g Repression by continuous growth hormone administration Mol Endocrinol 1990,4 53-8

Ziegler RG, Morris LE, Blot WJ, Pottern LM, Hoover R, Fraumine F Esophagal cancer among black men in Washington DC II J Natl Cancer Inst 1981,67 1199-1206

Zimmerman HJ Effects of alcohol on other hepatotoxins Alcoholism Clin Exp Res 1986,10 3-15

Zorn H Karzinom der ableitenden Harnwege bei Teer-, Asphalt- und Bitumen-arbeitern Zentralbl Arbeitsmed Arbeitssch Prophyl 1966,16 366-71

De Zwart WM Alcohol, tabak en drugs in cijfers Utrecht Nederlands Instituut voor Alcohol en Drugs, 1989

CHAPTER II

GROWTH AND LIVER MORPHOLOGY AFTER LONG-TERM ETHANOL CONSUMPTION OF RATS

J.A.G. van de Wiel, C.M.P. Duijf, J.C.L.M. Pertijs,
J.H.J. Copius Peereboom-Stegeman, R.P. Bos.

Department of Toxicology, Faculty of Medical Sciences,
University of Nijmegen, P.O. Box 9101,
6500 HB Nijmegen
The Netherlands

Laboratory Animals 1990;24:265-272

SUMMARY

Ethanol was administered to female and male Wistar rats by mixing it with their drinking water. Ethanol concentrations were gradually increased up to either 8% or 15%. Female rats receiving 8% ethanol in their drinking water consumed 5-13 g, males 4-10 g daily. The ethanol/total food caloric intake percentages were 13 to 20% and 9 to 15% for female and male rats, respectively. There was no difference in body weight and relative liver weight between treated rats and their controls. Female and male rats receiving 15% of ethanol in their drinking water consumed 8-14 g ethanol per kg body weight per day. The percentages of ethanol/total food caloric intake were stabilised at about 25% for both sexes. Growth of the rats differed only slightly from controls; a tendency for a higher increase of body weight of the control rats was found. No difference in relative liver weight between ethanol-treated and control rats was observed. Microscopic examinations revealed that the ethanol treatment resulted in fat accumulation in the liver cells. A proliferation of the Smooth Endoplasmic Reticulum (SER) was more marked in the 15% dosed rats than in the 8% dosed rats and more distinct in female rats than in male rats in both dosage groups.

INTRODUCTION

Several methods of administration of ethanol to rats have been reported in the literature, varying from injection of daily doses (Farbiszewski *et al.*, 1988) to synthetic liquid diets (Lieber & DeCarli, 1967, 1982; French, 1968). Ethanol is often added to the drinking water (Nadkarni & D'Souza, 1988; Prasad *et al.*, 1985; Benedetti *et al.*, 1988; Väänänen & Lindros, 1985; Liu *et al.*, 1975; Dobbins *et al.*, 1972; Thorpe & Shorey, 1965).

In this paper a method of administering ethanol to rats via the drinking water is described. A dosage procedure is developped that corresponds to 'moderate' drinking in humans. Moderate drinking is defined as long term consumption of a maximum of 20% of the daily caloric intake as ethanol (Prasad *et al.*, 1985; Zentella de Pina *et al.*, 1989). Treated animals should have similar weight gain and caloric intake as controls. No severe hepatocellular damage should be observed, although liver enzymes could be induced.

MATERIALS AND METHODS

Animals

Homebred Cpb:WU (Wistar) rats were used. The initial weights varied between 100-120 g (females) and 120-150 g (males). The rats were about 6 weeks old.

They were of specified pathogen free quality (antibodies against *Pneumonia* virus of mice and the pin-worm *Syphacia muris* were found, no other pathogens). Rats were kept in Macrolon type 3 cages on sterilized softwood granules as bedding with 3 animals per cage. Rats were housed in stainless steel metabolism cages on a wire mesh for measurements on individual rats The rats were weighed three times a week

The animals were provided with RMH-TM pellets (Hope Farms b v., Woerden, The Netherlands). Groups of 12 rats were used in each experiment (six females and six males) Three rats of each sex were ethanol-treated, the other three received tap water The ethanol-treated rats had free access to water with an increasing ethanol percentage reaching 8 or 15% (called the 8% and 15% ethanol treatments) after 2 and 3 weeks respectively Details of the ethanol treatment schemes are given in table 1.

Room temperature was regulated (22 ± 2°C), relative humidity varied between 40 and 60% The animals were exposed to artificial light between 1900 h and 0700 h so that blood ethanol concentrations could be measured during the active period of the rats Blood samples were taken from the rats via a canula in the *arteria femoralis* The animals did not need to be anaesthetized using this method

8% ethanol treatment	4 days 2% (w/v) ethanol in tap water 3 days 4% (w/v) ethanol in tap water 7 days 6% (w/v) ethanol in tap water 28 days 8% (w/v) ethanol in tap water
15% ethanol treatment	4 days 2% (w/v) ethanol in tap water 3 days 4% (w/v) ethanol in tap water 4 days 6% (w/v) ethanol in tap water 3 days 8% (w/v) ethanol in tap water 4 days 10% (w/v) ethanol in tap water 3 days 12% (w/v) ethanol in tap water 21 days 15% (w/v) ethanol in tap water

Table 1 Scheme of ethanol treatment of rats

Ethanol consumption

The amount of ethanol consumed was calculated from the volumes of the ethanol/water mixture the rats drank and food pellets were weighed The percentage of the total caloric intake due to ethanol was calculated from these values.

Microscopy

The rats were killed by cervical dislocation at the end of the period of ethanol treatment. The livers were excised and weighed and the ratio liver to body weight was determined. The samples for light microscopy were fixed in McDowell (1% glutardialdehyde and 4% paraformaldehyde in 0.08M $NaH_2PO_4.H_20$ [pH=7.4]) at room temperature for at least 24 hours. They were embedded in paraplast. Sections of 6 μm were cut on a Leitz microtome and stained with a hemateine-eosine mixture. Samples for electron microscopy were fixed in 2% glutardialdehyde in 0.1 M phosphate buffer (pH=7.4) at 4C for 24 hours and were embedded in EPON 812. Semithin (1 μm) sections for light microscopy were cut on a Reichert ultra microtome OMU3. These were stained with toluidine blue. Ultrathin sections of 60 nm were contrasted with uranylacetate and lead citrate. They were studied in a Philips EM301 Electron Microscope at 60 KV.

Chemicals

The ethanol used was Ethanol absolute GR supplied by Merck (Darmstadt, Germany). The ethanol in blood concentrations were determined using the Boehringer testkit Cat no. 176290 (Boehringer Mannheim GmbH, Mannheim, W. Germany).

EPON 812, paraplast and stains were supplied by Merck (Darmstadt, Germany). Leadnitrate was obtained from Analar BDH Chemicals Ltd. (Poole, England).

Statistical analysis

Results are presented as mean values. Statistical analysis was carried out using the SAS (Statistical Analysis System) package on a VAX785 minicomputer. The procedures used were ANOVA for analysis of variance with equal cells and GLM (general linear model) for analysis of variance with unequal cells.

RESULTS

Blood ethanol concentrations

Ethanol concentrations in the blood of 3 female and 3 male rats consuming 8% ethanol in their drinking water varied widely between the animals (figure 1).

There was also great variability in the values measured during one day. Comparison of the data from several days did not show a consistent pattern in the values for individual rats. The minimum blood ethanol value measured was 0 g/l, the maximum value was 0.9 g/l.

A blood sample was taken at time intervals of 4 hours from the rats and the volume of water drunk was measured. The blood ethanol concentration was 0 g/l when a rat did not drink the ethanol/water mixture and greater than 0 g/l when a rat drank. However, there was no quantitative relationship between the amount

of ethanol/water mixture drunk during 4 hours and the blood alcohol levels at the end of that period.

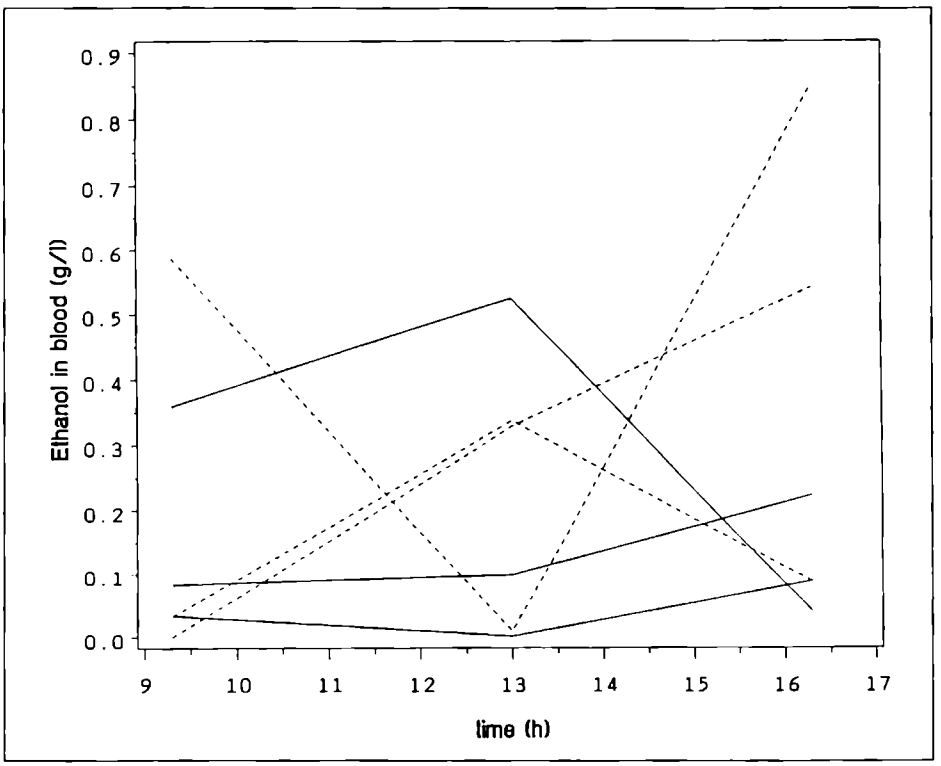


Figure 1. Blood ethanol concentrations. Ethanol concentrations measured three times during a dark period in blood of three female and three male rats from the 8% ethanol treatment group. (—) ; female rats, (----) ; male rats).

Ethanol consumption and growth

The daily ethanol consumption of rats receiving the 8% treatment is shown in figure 2a. Female rats consumed more ethanol than male rats; 5-13 and 4-10 g/kg/day respectively. Rats receiving the 15% ethanol treatment consumed 6-14 g/kg/day for both, female and male rats (figure 2b).

The ethanol consumption as a fraction of total food caloric intake during the 8% ethanol treatment is shown in figure 3a. Levels of 13 to 20% and 9 to 15% for female and male rats, respectively were attained. In the 15% ethanol treatment group the level stabilized in the last two weeks of ethanol-treatment at about 20% of total caloric intake for both female and male rats (figure 3b).

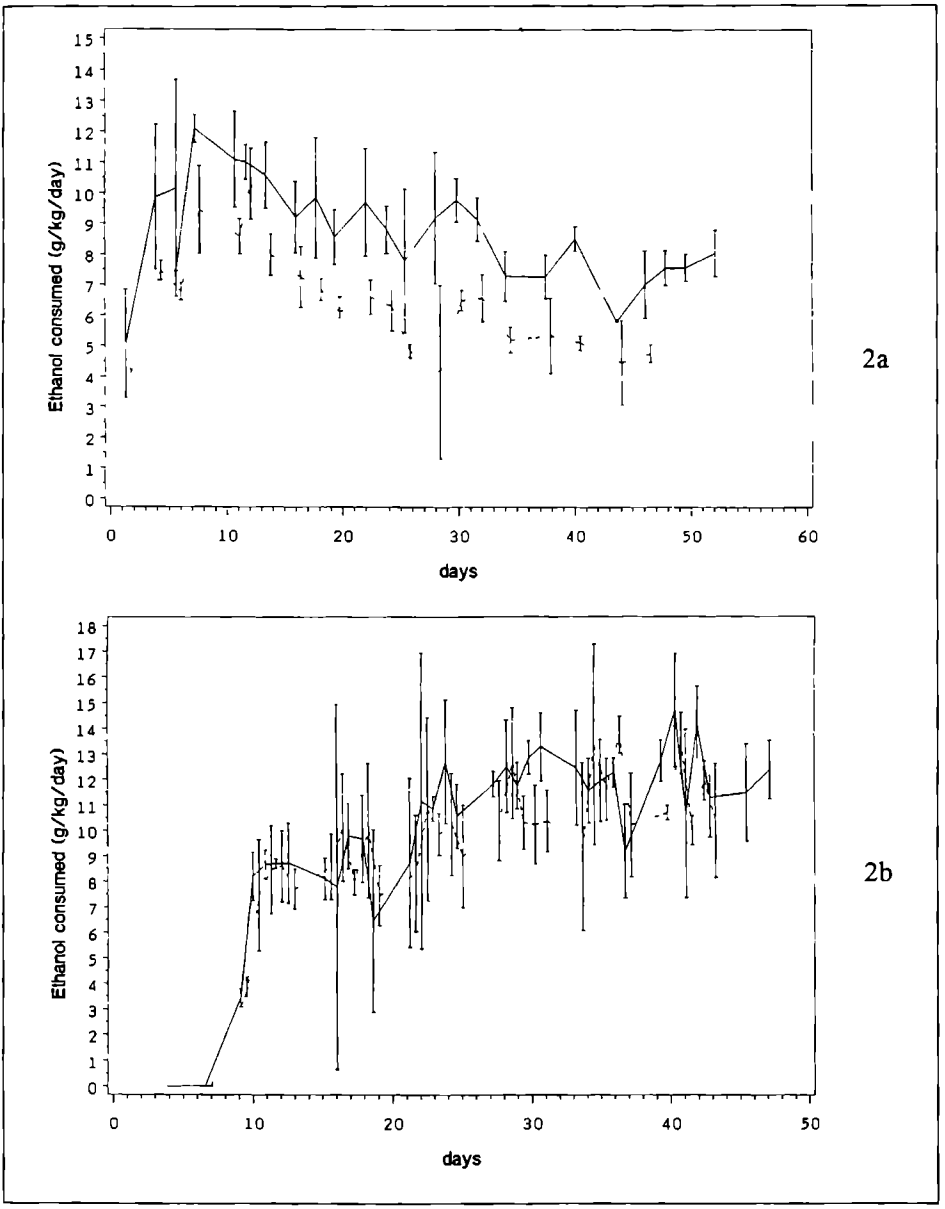


Figure 2 Amount of ethanol consumed The amount of ethanol (g/kg/day) consumed by female (—) and male (-----) rats with the 8% ethanol treatment (a) or 15% ethanol treatment (b) Each point represents the mean (± SD) of three rats

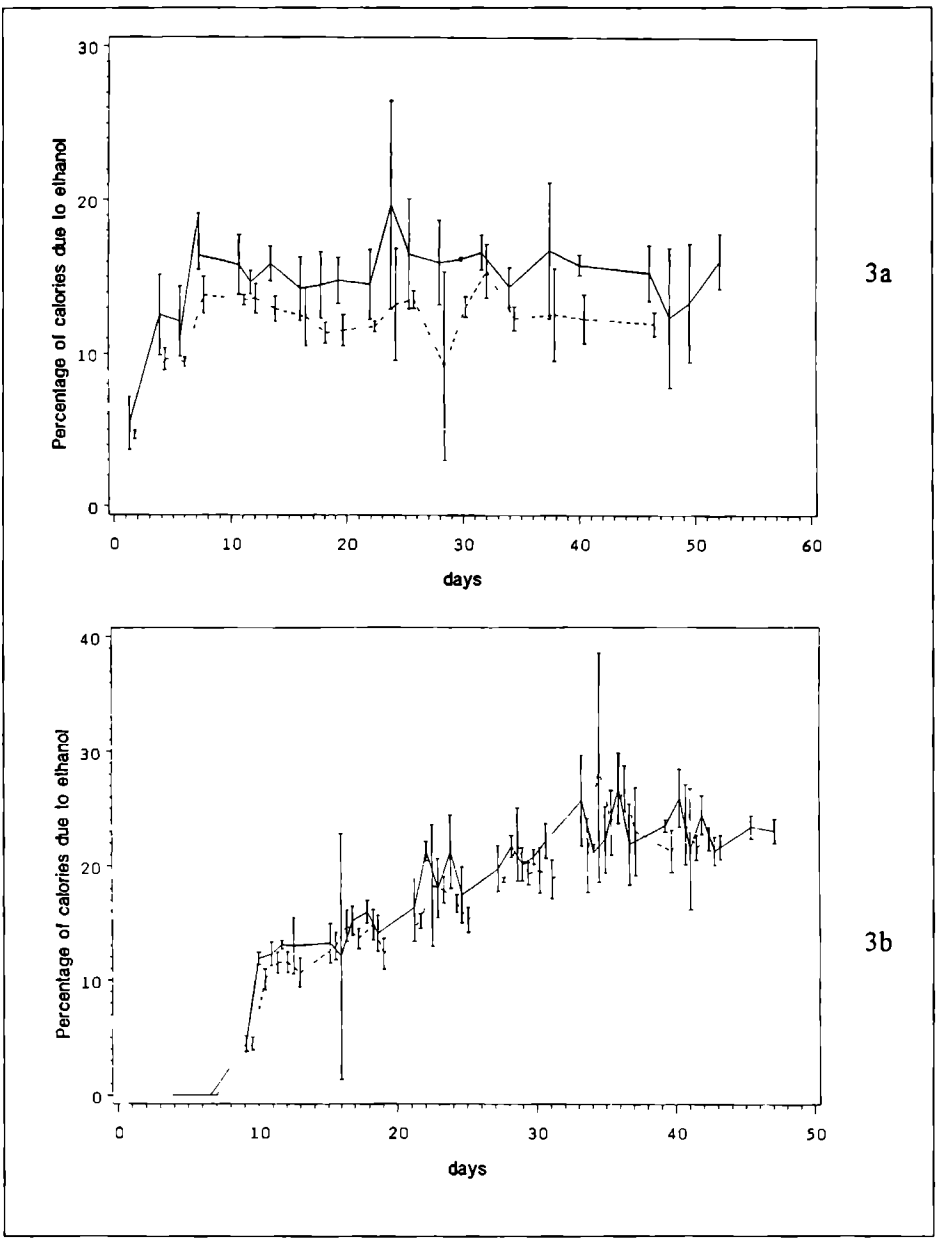


Figure 3 Relative caloric intake Ethanol consumption as a percentage of total caloric intake during the 8% ethanol treatment (a) or 15% ethanol treatment (b) Each point represents the mean (±SD) of three female (—) or three male (----) rats.

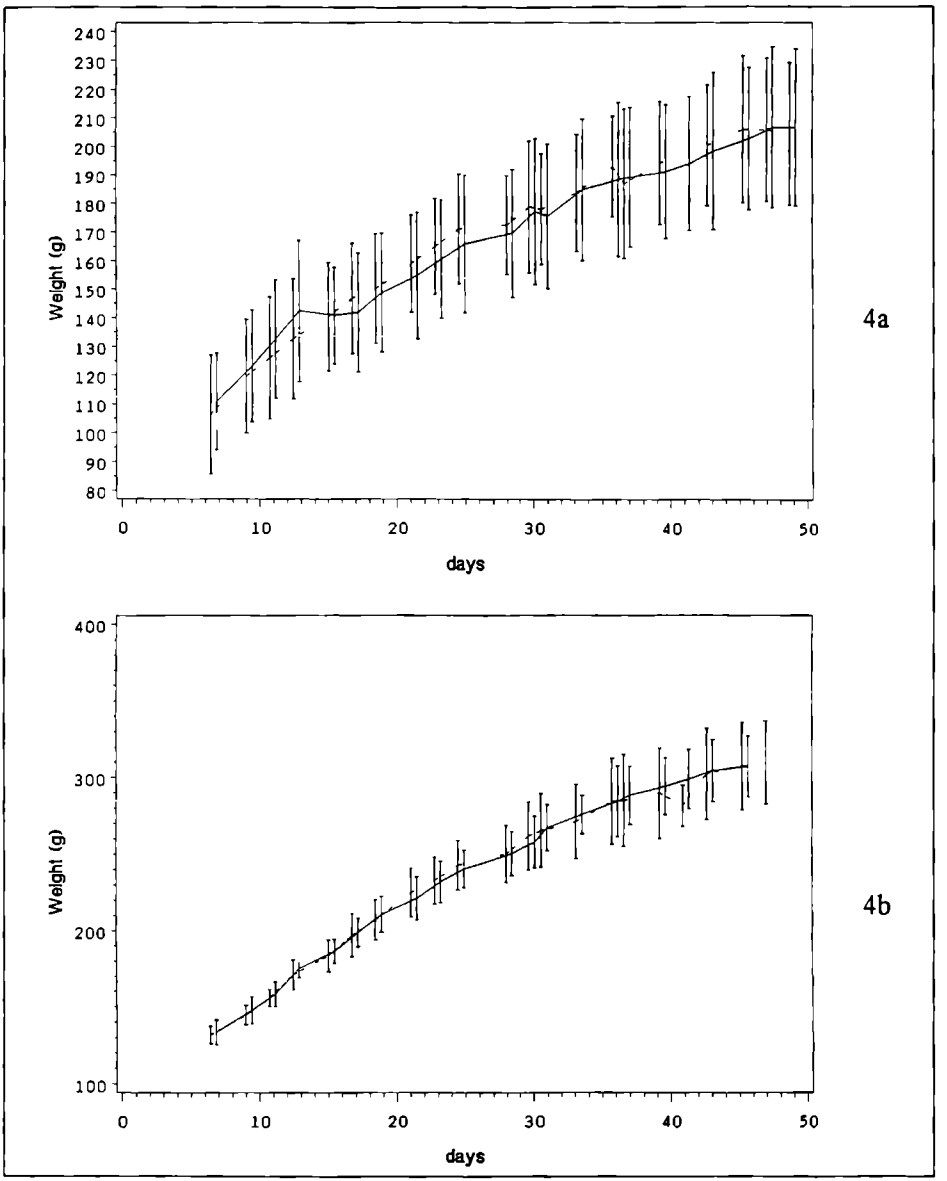


Figure 4 Growth of rats Weights of female rats drinking only water (-----) or with the 8% ethanol treatment (a) or with the 15% ethanol treatment (c) (——) Weight of male rats drinking only water (-----) or with the 8% ethanol treatment (b) or with the 15% ethanol (d) treatment (—·—) Each point represents the mean (±SD) of three rats

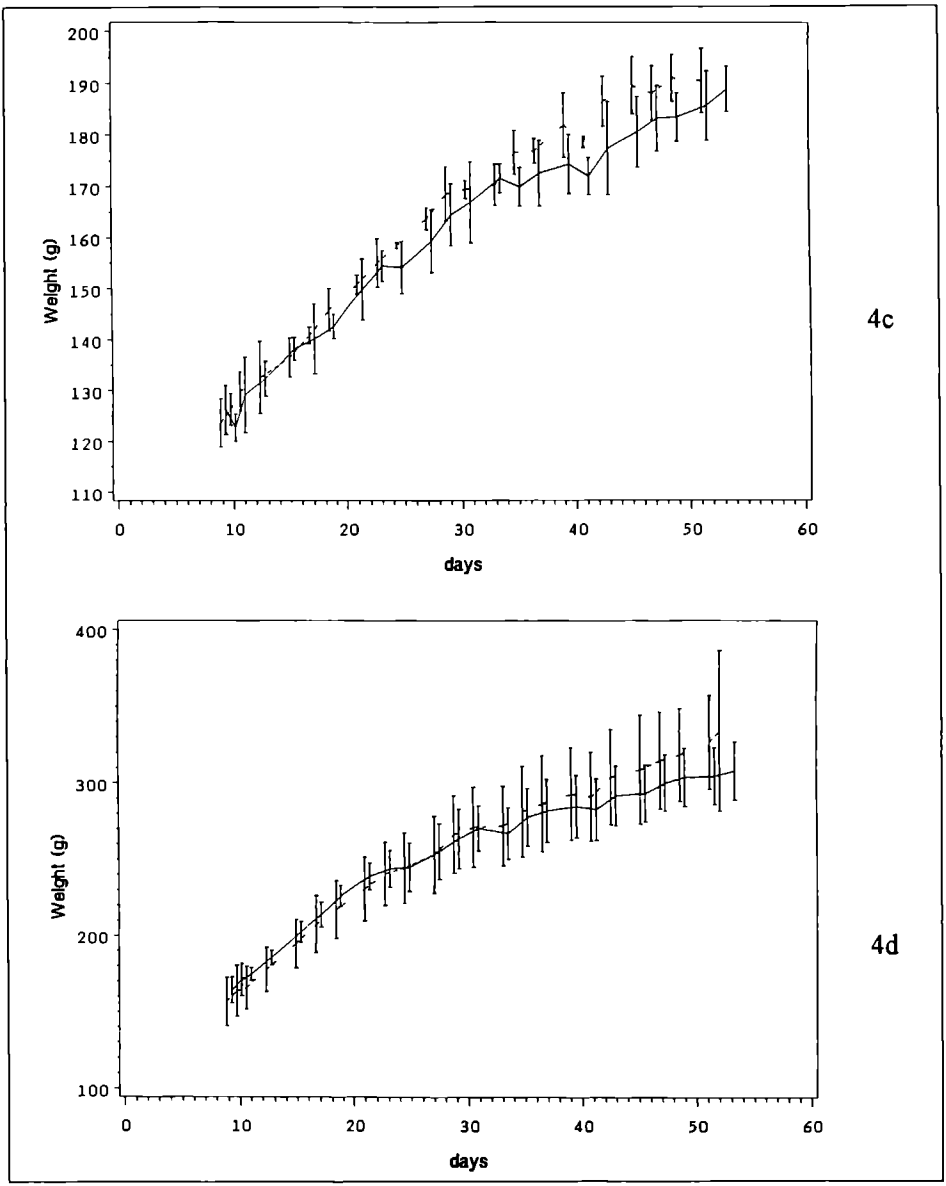


Figure 4 Growth of rats Weights of female rats drinking only water (——) or with the 8% ethanol treatment (a) or with the 15% ethanol treatment (c) (——) Weight of male rats drinking only water (----) or with the 8% ethanol treatment (b) or with the 15% ethanol (d) treatment (——) Each point represents the mean (±SD) of three rats

The body weights of female and male rats during the 8% ethanol treatment period were compared with controls (figures 4a and 4b). Growth rate of ethanol-treated and control rats was equal (differences were not statistical significant). The body weights of female and male rats during the 15% ethanol treatment period were slightly lighter than the control rats and grow more slowly (figures 4c and 4d) but this difference was not statistically significant.

Relative liver weight

There were no statistically significant differences in liver weights expressed as a percentage of the total body weight between ethanol and non-ethanol treated rats using analysis of variance. There was also no statistical significant difference in the relative liver weights between the two sexes (table 2).

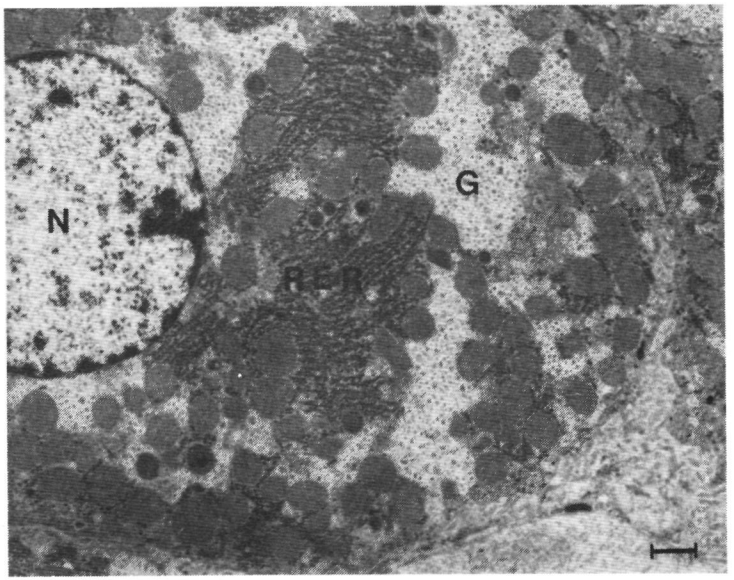
Percentage ethanol	Sex	N	Relative liver weight (%)	SD
0	F	20	4.1	0 6
0	M	21	4.1	0 4
8	F	9	3.9	0 2
8	M	9	4.0	0.4
15	F	12	3.8	0.5
15	M	12	4.1	0 5

Table 2 Relative liver weight of rats after 6 weeks on different diets (liver weight/body weight) N=number of rats, SD standard deviation

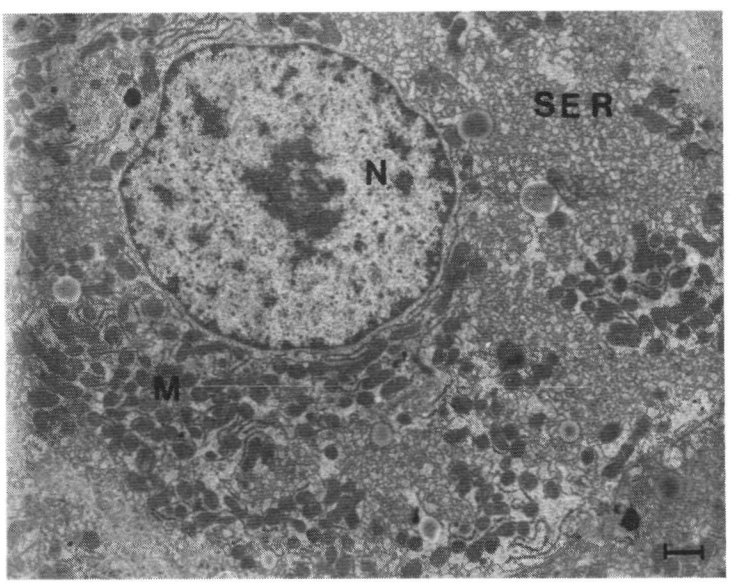
Microscopic examination

The livers of ethanol-treated and control animals appeared under light microscopy to have a rather homogeneous structure. Fat droplets were observed in the hepatocytes of ethanol treated rats but little glycogen.

Electronmicrographs of the liver of rats on the ethanol diet showed an accumulation of fat droplets especially near the sinusoides. The proliferation of the Smooth Endoplasmic Reticulum (SER) was clearly seen; the nuclei were often lobed and sometimes possessed clumped chromatin. The Rough Endoplasmic Reticulum (RER) did not appear swollen and ribosomes were still attached to the membrane. The number of mitochondria in ethanol-treated rats was not increased and their appearance not changed; giant mitochondria and crystalline enclosed particles were not found. No bile obstruction was observed. The effects on the liver described above were more obvious in female rats than in male rats; as well as more obvious in the 15% ethanol group than in the 8% ethanol group (figure 5a and 5b).



5a



5b

Figure 5. Electronmicrographs of rat liver. Electronmicrograph of a control female rat liver (a) and of a liver of a female rat from 15% ethanol treatment (b). N=nucleus, RER=Rough Endoplasmic Reticulum, SER=Smooth Endoplasmic Reticulum, G=glycogen, M=Mitrochondrion. (Magnification 5a-7100, 5b=6100 |-|=1μm) .

DISCUSSION

A common method of chronic exposure of rats to ethanol is the feeding of a liquid diet containing essential dietary components supplemented with ethanol. This diet produces a reproducible animal model for the study of several pathological events observed in 'heavy' drinking humans (Lieber & DeCarli, 1967) Another method is the addition of ethanol to the drinking water, with the rats obtaining their protein, fat, carbohydrate, vitamins and minerals from food pellets. The results obtained with the two kinds of diets differ greatly, not just because of the use of different treatment methods but also because of differences in rat strains, periods of treatment and amount of fat, protein and carbohydrate in the diet. Lieber & DeCarli, (1973) observed fat deposition in the liver after feeding the liquid diet. They compared an original diet containing 35% fat with a low fat diet (12%) and found that livers were less fatty with the same ethanol ingestion in the low fat diet (Lieber and DeCarli, 1982).

Several investigators have proposed modifications to the liquid diet. A higher content of lipotrophic factors and amino acid nitrogen (Porta *et al.*, 1968) produced less steatosis, although swelling of mitochondria and loss of cristae organisation was observed by Banks *et al.* (1969). Neither chemical nor histological evidence of liver fat accumulation was found with the AIN-76A standard diet (Bieri *et al.*, 1977) containing 21% of protein, 12% of fat and 30% of carbohydrate (Schoemaker & Visek, 1988).

The importance of the carbohydrate content of the diets was emphasized by Rao *et al.*, 1987. They found that rats consumed more diet and grew faster when the concentration of ethanol in the liquid diet was decreased from 36% to 20%, and the carbohydrate content was increased. A lower fat content of the liver was found. The rats consumed an equal amount of ethanol-derived calories per day with both diets.

The food pellets used in our study contained 22.4% of the energy content as protein, 10.8% as fat and 49.3% as carbohydrates. They, therefore formed a rather low fat, high carbohydrate diet.

The absolute amount of ethanol ingested in g/kg/day and the percentage of the total caloric intake due to ethanol seem good parameters to determine and compare the ethanol dose due to the different diets. The rats consumed 36% of their daily caloric intake as ethanol using the method of Lieber and DeCarli (1982). This is equivalent to an ethanol intake of 12-18 g/kg/day. Blood ethanol levels varied between 1.0 and 1.5 g/l. The ethanol intake in absolute amounts was comparable with the results of our study although the percentage of total caloric intake due to ethanol by our rats was lower; 20% compared with 36%. In our study only the blood ethanol levels in rats receiving the 8% ethanol treatment were measured and these were rather low.

Rubin *et al.* (1970) described morphological changes such as hepatic steatosis, proliferation of hepatic SER, increased microsomal protein and enlarged mitochondria using the Lieber/DeCarli diet. In our study steatosis and proliferation of the SER was also observed. These also have been observed by other investigators giving rats ethanol in their drinking water with other nutrients in food pellets. Benedetti *et al.* (1988) observed areas of proliferated SER in hepatocytes containing lipid droplets of various size. They also observed bizarre shaped mitochondria. Their laboratory animal diet contained 41% of calories as carbohydrate, 21% as protein and 6% as fat. The chronic ethanol group received ethanol solution supplemented with 6% sucrose. The ethanol concentration was gradually increased from 5% (v/v) to 25% (corresponding to a mean daily ethanol intake of 14.8 g/kg), which was reached on the 29th day. Two control groups were used; one with 6% of the calories as sucrose and one with tap water. Blood ethanol levels reached a level of 1.1 g/l in the fifth week. Dobbins *et al.* (1972) performed a morphometric analysis of the effects of ethanol upon rat liver. They compared three diets in their study; chow with 25% of calories as ethanol in the drinking water, chow with isocaloric sucrose and chow supplemented with 2% choline plus 25% ethanol. In the 25% ethanol group they observed an increased hepatic cell size, an increased total volume of mitochondria and many lipid droplets. These phenomena were partially reversed by choline supplementation. The surface area of both SER and RER was reduced to 50% in the ethanol treated animals. The diets consisted of 19% of the calories as protein, 7.5% as fat and 43.5% as mixed starches.

Thorpe and Shorey (1965) added ethanol to the drinking water with a final concentration of 20%. Their diet was also low in fat and high in carbohydrate. The control group received diet and plain water ad libitum. The ethanol dose was equivalent to 5.7 g/kg/day; ethanol providing 22% of the total caloric intake. No change in liver lipid content was observed.

Most diets in which normal food pellets are used in combination with ethanol in drinking water are low in fat content and high in carbohydrate. The ethanol dose has to be at least 15% to lead to observable damage. The method we have described and chosen is relatively simple and led to a considerable intake of ethanol (5-13 g/kg/day) which is 20% of the daily caloric intake.

Electron microscopy of the rat livers confirmed proliferation of SER as an effect of ethanol as previously described by Rubin *et al.* (1968). The livers of female rats which received 15% ethanol in their drinking water contained more fat droplets than the livers of male rats. Small fat droplets were observed in the livers of the rats that received 8% ethanol. No effects on mitochondria nor severe pathological effects such as liver necrosis or cirrhosis were observed.

The growth and development of the rats was normal during the treatment based on the body weight curves. The ratio of liver weight to body weight did not differ significantly between ethanol-treated and control rats.

Our results confirm that addition of ethanol to the drinking water enabled the ingestion of high amounts of ethanol, 5-13 g/kg/day which is comparable to the Lieber/DeCarli diet This resulted in a considerable caloric intake from ethanol (12-20%) Human alcoholics are defined as those who are chronically consuming at least 20% of their daily caloric intake as ethanol (Lelbach, 1974) This rat model therefore seems realistic We think that this model is suitable for further studies on the effects of long term ethanol exposure on the toxicokinetics of xenobiotics

REFERENCES

Banks WL, Kline ES, Higgins ES (1969) Composition and Metabolism after Ethanol Consumption in Rats Fed Liquid Purified Diets Journal of Nutrition 100 581-594

Benedetti A, Brunelli E, Risicato R, Cilluffo T, Jezequel AM, Orlandi F (1988) Subcellular changes and apoptasis induced by ethanol in rat liver Journal of Hepatology 6,137-143

Bieri JG, Stoewsand GS Briggs GM, Phillips RW Woodard JC, Knapta JJ (1977) Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies Journal of Nutrition 107,1340-1348

Dobbins WO Emory MD, Rollins L Brooks SG (1972) A quantitative morphological analysis of ethanol effect upon rat liver Gastro enterology 62(5) 1020-1033

Farbiszewski R, Holownia A, Chwiecko M (1988) Changes in activities of some Ammonia-Metabolizing Enzymes in the rat liver and the brain after chronic ethanol administration Biochemical Medicine and Metabolic Biology 40 219-224

French SW (1968) Fragility of liver mitochondria in ethanol-fed rats Gastroenterology 54(6),1106-1114

Lelbach WK (1974) in Research Advances in Alcohol and Drug Problems (Eds Gibbins RJ, Israel Y, Kalant H, Propham RE, Schmidt W & Smart RG) 1,93 New York John Wiley

Lieber CS & DeCarli LM (1967) Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet Journal of Nutrition 91 331-336

Lieber CS & DeCarli LM (1973) Alcohol, nutrition and the liver The American Journal of Clinical Nutrition 26,1163-1165

Lieber CS & DeCarli LM (1982) The feeding of alcohol in liquid diets Two decades of applications and 1982 update Alcoholism Clinical and Experimental Research 6(4) 523-531

Liu SJ, Ramsey RK, Fallon HJ (1975) Effects of ethanol on hepatic microsomal drug-metabolizing enzymes in rat Biochemical Pharmacology 24,369-378

Nadkarni GD & D'Souza NB (1988) Antioxidant and free radical scavenging enzymes in chronically ethanol consuming rats controversy over hepatic lipid peroxidation Drug and Alcohol Dependence 22,161-164

Porta EA, Doch OR, Comez-Dumm CLA & Hartroft WS (1968) Effects of dietary protein on the liver of rats in experimental chronic alcoholism Journal of nutrition 94,437-446

Prasad JS, Crankshaw DL, Erickson RR, Elliott CE, Husby AD, Holtzman JL (1985) Studies on the effect of chronic consumption of moderate amounts of ethanol on male rat hepatic microsomal drug metabolizing activity Biochemical Pharmacology 34(19),3427-3431

Rao GA, Riley DF & Larkin EC (1987) Dietary carbohydrate stimulates alcohol diet ingestion, promotes growth and prevents fatty liver in rats Nutrition Research 7,81-87

Rubin E, Hutterer F, & Lieber CS (1968) Ethanol increases hepatic smooth endoplasmic reticulum and drug-metabolizing enzymes Science 159,1469-1470

Rubin E, Bacchin P, Gang H & Lieber CS (1970) Induction and inhibition of hepatic microsomal and mitochondrial enzymes by ethanol. Laboratory Investigations 22(6),569-580

Schoemaker JD & Visek WJ (1988) Growth, liver lipid and blood amino acids in rats fed ethanol with an adequate diet. Drug and Alcohol Dependence 12;49-54.

Thorpe MEC & Shorey CD (1965) Long term alcohol administration Its effects on the ultrastructure and lipid content of the rat liver cell American Journal of Pathology 48(4);557-576.

Vaänänen H & Lindros KO (1985) Comparison of ethanol metabolism in Isolated Periportal of Perivenous Hepatocytes. Effects of chronic Ethanol Treatment. Alcoholism: Clinical and Experimental Research 9(4);315-321.

Zentella de pina M, Villalobos-Molina R, Saavedra-Molina A, Riveros-Rosas H & Pina E (1989) Effects of moderate chronic ethanol consumption on rat liver mitochondrial functions. Alcohol 6;3-7

CHAPTER III

EXCRETION OF BENZO(*A*)PYRENE AND METABOLITES IN URINE AND FECES OF RATS: INFLUENCE OF ROUTE OF ADMINISTRATION, SEX AND LONG-TERM ETHANOL TREATMENT

J.A.G. van de Wiel, P.H.S. Fijneman, C.M.P. Duijf, R.B.M. Anzion, J.L.G. Theuws, R.P. Bos

Department of Toxicology, Faculty of Medical Sciences,
University of Nijmegen, P.O. Box 9101,
6500 HB Nijmegen
The Netherlands

Toxicology 1993;80:103-115

SUMMARY

The urinary and fecal excretion of benzo(*a*)pyrene [B(*a*)P] and its main metabolites were studied after oral and intraperitoneal administration of B(*a*)P to male and female ethanol-treated and non-ethanol-treated rats. After oral administration of B(*a*)P more mutagenic compounds as well as B(*a*)P metabolites were found in feces than after intraperitoneal administration. The excretion of B(*a*)P metabolites in urine and feces after oral administration were maximal at days 1 and 2 whereas after intraperitoneal administration excretion was maximal at days 2 and 3. In males, the amounts of excreted phenolic metabolites in urine and feces were generally higher than in females. The amounts of mutagenic products in urine and feces of males were also higher than in females after intraperitoneal and oral administration of B(*a*)P. In urine of female rats that received B(*a*)P intraperitoneally, a decreased excretion of phenolic metabolites was found after ethanol treatment. In feces of both male and female rats, a decreased excretion of 3-hydroxy-B(*a*)P was found after ethanol treatment. In this study, the influence of sex and administration route on the excretion of B(*a*)P metabolites was more pronounced than the effect of ethanol treatment.

INTRODUCTION

The impact of human life-style factors on uptake, distribution, biotransformation and excretion of xenobiotics is clearly recognized. The main, non-work related, life-style associated parameters are alcohol use, smoking, drug and alimentary habits (Fournier & Thomas, 1986). Interactions between alcohol use and pharmacokinetics of Polycyclic Aromatic Hydrocarbons (PAHs) are of interest because PAHs are ubiquitous environmental pollutants produced by common industrial and transportational sources and tobacco smoking. Alcohol use is a widespread life style factor. The risk of cancer of the larynx, pharynx and oesophagus was additive or multiplicative for people that smoke and drink alcohol (IARC, 1988). Effects of ethanol on the organism are dependent on the amounts of ethanol consumed. Consumption of 15-20% of the total daily caloric intake is defined as 'moderate' drinking in humans (Prasad *et al.*, 1985). We have developed an animal model in which rats receive such amounts of ethanol (van de Wiel *et al.*, 1990).

Several researchers have investigated the pharmacokinetics of PAH in laboratory animals by analyses of excretion products. Mostly benzo(*a*)pyrene [B(*a*)P] is used as a model substrate for the study of the pharmacokinetics of PAH. Elimination of B(*a*)P proceeds via the bile. Foth *et al.*, (1988) reported that 42.5% of an i.v. dose of [^{3}H]B(*a*)P appeared in the bile as total radioactivity during the first 4 h. Only 2% of this dose was excreted in the urine in 36 h. After a subcutaneous injection

of [^{3}H]B(*a*)P, it was found that the feces contained the highest amounts of radioactivity, which were 2-4 fold higher than the amounts found in urine (Uziel & Haglund, 1988).
The biological availability of PAH is expected to be dependent on the route of administration. Grimmer *et al.* (1988) found that after oral application of chrysene 60% of the dose was recovered as excretion products in urine and feces (1.5% and 72.3%, respectively), while after an i.p. application this was only 5% (urine 0.55%, feces 5.6%). After oral and intraperitoneal administration of pyrene, Jacob and Brune (1989) found 53.4% and 3.1% as pyrene (metabolites) respectively, in urine and feces.
Sex differences in the hepatic metabolism of drugs in the rat have been reported by several investigators. Gurtoo and Parker (1977) measured a sex dependent regulation of B(*a*)P metabolism in rat tissues. Aryl Hydrocarbon Hydroxylase (AHH) activity in hepatic microsomes was 4-6 fold greater in male rats compared with female rats. No differences in AHH activity were seen in the lung and spleen of male and female rats. AHH activity in kidneys from female rats was 86% higher than that of male rats. We wondered whether in vitro measured sex differences in PAH metabolism would be observed in in vivo excretion profiles of B(*a*)P and metabolites in urine and feces.
In this study benzo(*a*)pyrene [B(*a*)P] is used as a model substrate for the study of the influence of several parameters on the excretion of PAH. The excretion of B(*a*)P and metabolites in urine and feces of ethanol-treated rats and control rats were studied after oral (p.o.) and intraperitoneal (i.p.) administration of B(*a*)P. B(*a*)P-metabolites excreted in male and female rats were compared to detect sex differences and the influence of ethanol treatment.

METHODS

Animals
Homebred Cpb:WU (Wistar) rats were used. The initial weights varied between 100-120 g (females) and 120-150 g (males). The rats were about 6 weeks old when the ethanol treatment started and about 3 months at the time of the B(*a*)P administration. They were of specified pathogen free quality (antibodies against *Pneumonia* virus of mice and the pin-worm *Syphacia muris* were found, no other pathogens). Rats were kept in Macrolon type 3 cages on sterilized softwood granules as bedding with 3 animals per cage. At the time of the in vivo experiments the rats were housed in stainless steel metabolism cages designed for the separate collection of urine and feces. The animals were provided with RMH-TM pellets ad libitum (Hope Farms B.V., Woerden, the Netherlands). Four groups of 12 rats were used; two groups of males and two groups of females. One group of males and one group of females received B(*a*)P intraperitoneally, the

others orally. In each group six rats were treated with ethanol, the other six received tap water. The ethanol-treated rats had free access to water with an increasing ethanol percentage reaching 15% w/v after 3 weeks. Ethanol treatment continued during B(*a*)P administration. Details of the ethanol treatment were described previously (van de Wiel *et al.*, 1990).
Room temperature was regulated (22 ± 2°C), relative humidity varied between 40% and 60%. The animals were exposed to artificial light between 0700 h and 1900 h.

Chemicals
Ethanol absolute GR was obtained from Merck, Darmstadt, Germany. Benzo(*a*)pyrene [B(*a*)P] was purchased from Sigma, St Louis, MO, USA, and was about 98% pure. B(*a*)P metabolites were obtained from the NCI Chemical Carcinogen Repository, Midwest Research Institute, Kansas city, MO, USA:
B(*a*)P-trans-9,10-dihydrodiol and B(*a*)P-trans-4,5-dihydrodiol purity was >99%, B(*a*)P-cis-7,8-dihydrodiol purity was >97%, 9-hydroxy-B(*a*)P purity was >96% and 3-hydroxy-B(*a*)P purity was >98% (determined by HPLC).
ß-glucuronidase/arylsulfatase (from Helix pomatia) was from Boehringer Mannheim GmbH, West-Germany. Aroclor 1254 was from Alltech Associates, Deerfield, IL, USA.

Experimental design
Groups of 12 rats received one dose of 1 mmol B(*a*)P/kg, either orally or intraperitoneally. A large dose of B(*a*)P was used because the metabolizing capacity of the rats was not known. Besides the dose had to be large enough dose to detect DNA adducts (not described in this manuscript). One ethanol-treated and one control rat were dosed with the vehicle (olive oil) only. Urine and feces were collected individually over periods of 24 h, one day before treatment, during four days after treatment and on the seventh day after B(*a*)P treatment. The ethanol-treated rats were continuously exposed to ethanol in their drinking water throughout the experiment. Urine and feces of days four and seven of the females that received B(*a*)P i.p. were not collected. Urine samples were adjusted with demineralized water to a volume of 20 ml urine per 200 g bodyweight of rats. This allowed a direct comparison of the metabolite concentrations and mutagenicity data of individual samples. After centrifugation about 10 ml was filter sterilized (0.2 µm from Millipore) for use in the Ames test. All samples were kept at -20°C until assayed.

Preparation of feces extracts
3 g of feces were boiled with 25 ml methanol under reflux for 60 minutes in a water bath of 70°C. The solid fraction was separated by centrifugation and reextracted with 15 ml methanol using ultrasonification for 10 minutes. After

filtration of this extract over glasswool both methanol extracts were pooled, and methanol was evaporated under nitrogen at 60°C. The residue was dissolved in 3 ml DMSO, ultrasonicated for 10 minutes and transferred into small glass vials until use (Willems & de Raat, 1985). If less than 3 g feces were produced, the residue was dissolved in DMSO at a final concentration of 1 g feces/ml DMSO. In a pilot study, the effectiveness of preliminary overnight incubation of feces with ß-glucuronidase/arylsulfatase was studied. No significant effect resulted from aryl sulfatase/ß-glucuronidase treatment. Consequently, it was not added to the standard analytical procedure. Either the ß-glucuronidase activity of the bacteria in colon and rectum is quite effective (and consequently the glucuronide conjugates are hydrolysed and found in feces as the deconjugated product), or the conjugates are hydrolysed during the methanol extraction. The ethylacetate extraction as performed by Bowes and Renwick (Bowes & Renwick, 1986) with or without enzymatic hydrolysis was less effective than the methanol extraction (data not shown). With the applied methanol extraction, the recovery of the metabolites from the feces were as follows; 3-hydroxy-B(*a*)P 92%, 9-hydroxy-B(*a*)P 95%, B(*a*)P-4,5-dihydrodiol 91%, B(*a*)P-7,8-dihydrodiol 94%, B(*a*)P-9,10-dihydrodiol 92%. In the Ames test, dilutions of the feces extract in DMSO were used. For HPLC analysis the feces extract was diluted to 5 mg/ml with methanol.

Pretreatment of urine for HPLC analysis

3 ml of a 24-hours urine sample were adjusted to pH=5.0 with 1.0 N HCl and buffered with 27 ml 0.1 M acetate buffer (pH=5.0). This solution was incubated overnight with 12.5 µl ß-glucuronidase/aryl sulphatase (1250 I.U.) at 37°C. A Sep-pak C18 cartridge (Waters, Milford, MA, USA) was used for the solid phase extraction of PAH metabolites. After priming the cartridge with 5 ml methanol and 10 ml distilled water, respectively, the hydrolysed sample was drawn through the cartridge at a rate of approximately 10 ml per min. Subsequently, the cartridge was washed with 10 ml distilled water. Retained solutes were eluted using 10 ml methanol. The solvent was evaporated at 60°C under nitrogen and the residue dissolved in 2.0 ml methanol (Jongeneelen *et al.*, 1984).

Reversed-phase HPLC analysis

Analyses were performed with a Kipp Analytica HPLC equipped with two solvent pumps (model 9208), a solvent programmer (model 9224), an automatic sampler (model 9209) and a column thermostat (model 9222). Using a sample loop, a 20 µl aliquot was injected onto a 150 x 4.6 mm ID Nucleosil C18 (5 µm) column. Column temperature was 40°C, flow 1.0 ml per min. The following solvent program was commenced: 5 min 90% solvent A (60% aqua pure, 40% methanol), a linear gradient to 90% solvent B (100% methanol) in 40 min. followed by 10 min 90% solvent B.

Fluorescence detection was performed with a Perkin Elmer LS-4 spectrofluorimeter. The program for excitatiòn and emission wave lenghts during each run is given in table 1. The retention times of 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P were 36 and 37.0 ± 0.5 min, respectively. The retention times of B(*a*)P-9,10-dihydrodiol, B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol and B(*a*)P were 19, 26, 27 and 43 minutes, respectively. Quantification was done by measuring peak heights and comparing these with a standard range. For analysis of urine the standards were prepared in a urine matrix and extracted following the same procedure as the urine samples. The recovery of metabolites from the urine matrix was about 45% for the concentrations used in the standard range.
The metabolites in the feces extracts were quantified using a standard range of the metabolites in methanol. Unconjugated metabolites had a recovery of more than 90%, B(*a*)P was recovered for about 85%.

time (min)	excitation (nm)	emission (nm)	metabolites detected
0	280	406	B(a)P-9,10-dihydrodiol
21	265	405	B(a)P-4,5-dihydrodiol, B(a)P-7,8-dihydrodiol
31	265	430	3-hydroxy-B(a)P, 9-hydroxy-B(a)P
41	296	407	B(a)P
58	C	C	

Table 1. Program of Perkin Elmer LS-4 detector

Ames test
The *Salmonella* mutagenicity test was performed with the *Salmonella typhimurium* strain TA98 as described by Maron and Ames (1983). This strain was subjected to quality control including checks for the presence of the strain characteristics and mutability by diagnostic mutagens like 2-aminofluorene and 4-nitro-O-phenylenediamine. Amounts of 0.3 ml of filter sterilized urine were used in the Ames test (Bos *et al.*, 1980).
The amount of mutants found were standardized for 20 ml of urine produced per rat per day, the rat having a bodyweight of 200 g. Thereto corrections were made (see below). Feces extract concentrations varied from 0-20 mg feces equivalents per plate. The highest concentration at which no direct toxicity was observed was used to determine the amount of mutants per mg feces. It was found that 0.5 mg/plate showed no toxic effects towards the bacteria. The Ames test was

performed with and without activation. When performed with activation, S9 mix and ß-glucuronidase were added. The S9 mix contained 100 µl of a hepatic 9000 *g* supernatant from Aroclor 1254 induced rats per ml. To detect mutagenic compounds that are inactive due to glucuronidation, ß-glucuronidase was applied to the top agar. Every sample was determined in triplicate. After 48 h incubation at 37°C, the revertant colonies were counted.
For adjustment of the different doses of B(*a*)P the rats received, and the different weights of feces they produced, the following formula was applied:

$$Mutagenicity = \frac{\text{extra induced revertants}}{\text{dosefactor}} \times \frac{\text{total weight feces}}{\text{weight feces plated}}$$

This means that the mutagenicity is defined as the revertants of TA98 induced by extract of 24 hours feces of a rat having a bodyweight of 200 g after a dose of 1 mmol B(*a*)P/kg.
-Extra induced revertants: Number of revertants due to feces extract on one day after the administration of B(*a*)P minus number of revertants due to feces extract before B(*a*)P administration.
-Dosefactor: Factor for adjustment of the different doses the rats received. (For a rat of 200g the factor=1, for a rat of 220g the factor=1.1 etc.)
-Total weight of feces: Total weight of feces produced on a certain day (mg).
-Weight feces plated: Equivalent of the amount of feces plated (mg) in the Ames assay.

Statistical analysis
Results are presented as average values. Statistical analysis was carried out using the SAS (Statistical Analysis System) package on a VAX6410 minicomputer. The procedure used was GLM (general linear model) for analysis of variance with unequal cells. The data of total excretion of mutagenicity and metabolites per rat were logtransformed before analysis, because of the very different amounts of excreted metabolites after p.o. and i.p. B(*a*)P administration. Of the intraperitoneally dosed females only urine and feces of the first 3 days after B(*a*)P administration were available for the test, so for statistical analysis only the totals of these days were compared for all groups of treated rats. A p-value <0.05 was considered to be of statistical significance.

RESULTS

B(a)P metabolites in urine
3-hydroxy-B(*a*)P was the predominant metabolite (up to 1500 nmoles in 5 days) in all groups of rats. Amounts of 9-hydroxy-B(*a*)P and of B(*a*)P-9,10-dihydrodiol

and B(*a*)P-4,5-dihydrodiol were 1/100 of 3-hydroxy-B(*a*)P (to 15 nmoles). The amount of B(*a*)P-7,8-dihydrodiol was about 1/1000 of 3-hydroxy-B(*a*)P.
The amounts of B(*a*)P and metabolites excreted in urine in 3 or 5 days are shown in tables 2a and 2b. The rats that received B(*a*)P intraperitoneally showed a maximum in the excretion of metabolites on days 2 and 3 (Data not shown). In general, after oral administration of B(*a*)P a maximum in the excretion of metabolites appeared on day 1. Male rats had more metabolites in their urine than female rats ($p<0.0001$). The total excretion over 3 days of B(*a*)P metabolites after i.p. administration of B(*a*)P in female rats was significantly lower in ethanol-treated rats in comparison with non-ethanol-treated rats. In male rats, no significant effect was observed in the excretion of B(*a*)P metabolites due to ethanol treatment.

Mutagenic metabolites in urine
The mutagenicity in urine after intraperitoneal and oral administration of B(*a*)P is shown in table 3. The intraperitoneally dosed rats showed a maximum in the excretion of mutagenic metabolites in their urine on days 2 and 3 after B(*a*)P administration. After oral administration of B(*a*)P the rats had a maximal excretion of mutagenic compounds in their urine on days 1 and 2 (data not shown). The male rats had a significantly higher excretion of mutagenic compounds in their urine compared with female rats after intraperitoneal and oral administration of B(*a*)P ($p=0.0001$). The difference between the sexes was largest in the i.p. dosed animals. There were no statistically significant differences between ethanol-treated and control rats measured within each group.

B(a)P metabolites and unmetabolized B(a)P in feces
The excreted amounts of B(*a*)P metabolites and unmetabolized B(*a*)P in feces are shown in tables 2a and 2b. After i.p. administration, the main metabolite excreted was 3-hydroxy-B(*a*)P. The average amount excreted during the period measured, in males and females, with or without ethanol treatment was 5 moles. After oral administration the amounts of B(*a*)P excreted, and also of its metabolites, were much higher than after intraperitoneal administration ($p=0.0001$). In the i.p. treated animals, maximal excretion was found on days two and three, whereas in the p.o. treated rats, maximal excretion was found on days one and two (data not shown). In general after i.p. or p.o. treatment 3-hydroxy-B(*a*)P, 9-hydroxy-B(*a*)P, B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol and B(*a*)P-9,10-dihydrodiol were higher in feces extracts of males compared to females. However, this was not significant for 9-hydroxy-B(*a*)P and the opposite was true for B(*a*)P-4,5-dihydrodiol in the non-ethanol group after i.p. B(*a*)P administration.

The excretion of 3-hydroxy-B(*a*)P was significantly lower in ethanol-treated rats in comparison with control rats after oral as well as after intraperitoneal treatment, in both males and females..

Metabolite	0% EtOH		15% EtOH	
	Male	Female	Male	Female
3-hydroxy-B(*a*)P* ** (µmol)	5 0 ± 0 8 (1 9%)	2 9 ± 0 4 (1 5%)	2 4 ± 0 2 (0 9%)	2 0 ± 0 4 (1 0%)
9-hydroxy-B(*a*)P (nmol)	39 1 ± 10 7	9 4 ± 3 0	14 9 ± 5 4	13 6 ± 3 3
B(*a*)P-4 5-dihydrodiol (nmol)	39 2 ± 14 3	63 4 ± 7 5	46 7 ± 6 8	44 1 ± 8 9
B(*a*)P-7,8-dihydrodiol* (nmol)	14 7 ± 13 1	nd	4 0 ± 3 7	nd
B(*a*)P-9 10-dihydrodiol* (nmol)	26 7 ± 6 1	13 2 ± 1 5	22 8 ± 6 1	9 0 ± 2 3
B(*a*)P (µmol)	0 38 ± 0 26	0 02 ± 0 009	0 08 ± 0 04	0 10 ± 0 01
Feces/µmol total	5 5 ± 1 0	3 0 ± 0 5	2 6 ± 0 2	2 2 ± 0 4
% of dose	2 0	1 6	1 0	1 1
Urine/nmol* total	1161 ± 210	38 ± 8**	1646 ± 400	19 ± 3

Table 2a Amounts of B(*a*)P and metabolites excreted during 5 days in feces and urine of rats after intraperitoneal B(*a*)P administration N = 5 or 6 Values are means ± Standard Error of the Mean (SEM) Of the females that were administered B(*a*)P i p , only urine and feces of days one, two and three were collected The percentage of the dose recovered is indicated When no value is added, this was smaller than 1 % of the dose

* Significant difference between male and female rats P<0 01

** Significant difference between ethanol-treated and non-treated rats P<0 01

Metabolite	0% EtOH Male	Female	15% EtOH Male	Female
3-hydroxy-B(a)P* ** (μmol)	33 6 ± 5 2 (11 2%)	19 0 ±1 8 (10 9%)	17 5±0 7 (5 8%)	14 6±3 4 (8 4%)
9-hydroxy-B(a)P (nmol)	492 5 ± 94 1	346 5±34 1	686 6±163 8	429 3±79 6
B(a)P-4,5-dihydrodiol* (nmol)	202 6 ± 30 5	83 0±13 3	175 8±28 1	66 1±5 4
B(a)P-7,8-dihydrodiol* (nmol)	153 9 ± 20 7	37 7±8 3	107 9±22 0	42 4±8 7
B(a)P-9,10-dihydrodiol* (nmol)	40 8 ± 4 5	25 8±1 7	35 9±7 8	32 5±3 2
B(a)P (μmol)	206 2 ± 30 5	83 8±10 9	172 1±24 4	66 7±8 3
Feces/μmol total	240 7 ± 35 7	103 3 ± 12 7	190 6±25 1	81 9±11 7
% of dose	80 2	59 4	63 5	11 7
Urine/nmol* total	309 ± 98	90 ± 21	290 ± 61	96 ± 23

Table 2b Amounts of B(*a*)P and metabolites excreted during 5 days in feces and urine of rats after oral B(*a*)P administration
N = 5 or 6 Values are means ± Standard Error of the Mean (SEM) Of the females that were administered B(*a*)P i p , only urine and feces of days one, two and three were collected The percentage of the dose recovered is indicated When no value is added, this was smaller than 1 % of the dose
* Significant difference between male and female rats P<0 01
** Significant difference between ethanol-treated and non-treated rats P<0 01

GROUP[a,b]			MUTAGENICITY FECES/1000[c]		REVERTANTS URINE[d] *
sex	B(a)P	diet	with* activation	without activation	
M	i p	0% EtOH	210 ± 51	28 ± 10	622 ± 65
M	i p	15% EtOH	149 ± 33	33 ± 8	428 ± 34
F	i p	0% EtOH	185 ± 16	89 ± 25	2557 ± 134
F	i p	15% EtOH	176 ± 50	74 ± 8	2549 ± 103
M	p o	0% EtOH	4162 ± 356	213 ± 38	603 ± 88
M	p o	15% EtOH	3116 ± 434	95 ± 26	600 ± 53
F	p o	0% EtOH	3978 ± 156	320 ± 192	1157 ± 87
F	p o	15% EtOH	4271 ± 402	251 ± 43	1146 ± 75

Table 3 Mutagenicity in feces and in urine
a Total amounts of five days measured are given for each group, however, of the females that received B(*a*)P i p , only samples of days 1, 2 and 3 were collected
b Each group consisted of five or six animals (mean values are presented)
c Mutagenicity

$$\frac{\text{extra induced revertants}}{\text{dosefactor}} \times \frac{\text{total weight feces}}{\text{weight feces plated}}$$

Amounts ± standard deviations (SD) are given
d Filter sterilized urine is used in the Ames test, 0 1 ml per plate
Amount of revertants are given (± SD)
* Significant difference between male and female rats P<0 01
** Significant difference between ethanol-treated and non-treated rats P<0 01

Mutagenic metabolites in feces

The mutagenicity of the feces extracts in the Salmonella mutagenicity test with and without the addition of an activating system is shown in table 3. Each plate contained an amount of feces extract equivalent to 0.5 mg feces. The mutagenicity values were calculated from the formula presented in the Materials and Methods section. The mutagenicity measured using the activating system was far greater than the mutagenicity when no activating system was added In the Ames test, B(*a*)P is mutagenic only when a liver S9-fraction is added.

The i.p. dosed rats showed a maximum excretion of mutagenic metabolites in their feces on days 2 and 3, and sometimes on day 4 (males) after B(*a*)P administration. The p.o. dosed rats showed a maximum excretion of mutagenic

compounds in their feces on days 1 and 2 (Data not shown). After intraperitoneal administration of B(*a*)P, the amounts of mutagens in the feces were considerably lower than after oral dosing in both sexes; approximately $200x10^3$ mutants versus $4000x10^3$ mutants measured with an activating system. Without an activating system this was on average $65x10^3$ versus $200x10^3$ mutants. The total mutagenicity of feces extracts of male rats was higher than that of female rats ($p=0.02$) when no S9-mix was used. There were no statistically significant differences between the ethanol-treated and non-ethanol-treated groups with respect to the total excretion of mutagenic compounds in the feces. However, the ethanol-treated female rats showed a tendency towards a decreased excretion of mutagenic compounds after oral B(*a*)P administration.

DISCUSSION

This paper presents a study of the excretion of benzo(*a*)pyrene and metabolites after oral and intraperitoneal administration in rats of both sexes. Moreover, the effect of chronic alcohol consumption was investigated.

Route of B(*a*)P administration was a major determining factor with respect to differences in excretion time and amounts of excreted products. Also sex differences occurred; males excreted larger amounts of certain metabolites than females. Statistically significant differences due to ethanol treatment were observed only with 3-hydroxy-B(*a*)P. After ethanol treatment, a smaller amount of this metabolite was found in feces.

Administration route

The administration route largely determined the total amount of excreted metabolites. Both after p.o. and i.p. treatment, much more metabolites were found in feces compared to urine. These results are in agreement with those of other investigators. With respect to the total amount of B(*a*)P-metabolites in rats, Uziel and Haglund (1988) found that excretion in feces was 3 times higher than in urine. Kawamura (1988) found a difference with a factor 20 after oral administration. Although the factors vary, it is clear that the excretion of B(*a*)P and metabolites mainly took place via the bile and feces. This is well known for compounds of high molecular weight like B(*a*)P metabolites (Kotin *et al.*, 1959; Chipman *et al.*, 1981).

The total amount of the B(*a*)P dose that was excreted in urine and feces in rats after oral administration was about 72% (63% unmetabolized B(*a*)P, 9% 3-hydroxy-B(*a*)P and 1% other metabolites). After oral administration of the PAH chrysene to rats, Grimmer *et al.* (1988) found 59.2% hydroxychrysene and 13.1% chrysene in urine and feces in 72 hours. After oral administration of pyrene, Jacob *et al.* (1989) found a total recovery in urine and feces in three days of 53.4%;

7.2% as pyrene and 46.2% as 1-hydroxypyrene. Although the total recovery of the dose is comparable, we found relatively more of the unmetabolized compound.

Non-polar compounds like unmetabolized B(*a*)P are only excreted as such in the bile in very small amounts after entering the systemic circulation (Smith & Bend, 1979; Takahashi *et al.*, 1977). We may conclude that, after oral administration, a large amount of B(*a*)P has not been absorbed. The low rate of absorption may be due to the high dose we gave (1 mmol/kg versus 10-400 μg given by other investigators). This could saturate the absorption capacity of the gut or form a bolus from which the B(*a*)P is not readily absorbed. Besides the relatively high excretion of unmetabolized B(*a*)P, metabolites and mutagenic products in feces were much higher after p.o. compared to i.p. B(*a*)P administration. The rate of excretion of B(*a*)P and metabolites was higher, as well. Grimmer *et al.* (1988), Jacob *et al.* (1989) and Egestad *et al.* (1987) found a maximal excretion on day 1 for both routes of administration, whereas we found a maximal excretion on days 2 and 3 after i.p. administration. Both larger and faster excretion of B(*a*)P metabolites could be due to a higher biological availability of B(*a*)P after oral administration. The absorption through the gut seems to cause a high liver load and B(*a*)P biotransformation in a short time. The small intestine may play a role in B(*a*)P metabolism after oral administration as well. Autoinduction in this tissue can already be measured within 12 hours after PAH administration (Aitio, 1974). Apart from that, other investigators reported that after i.p. administration at least 20% of the dose was not recovered with or without acid hydrolysis. This fraction most probably consists of glutathione conjugates or may be regarded as covalently bound (Egestad, 1987). Another factor that could reduce the availability of B(*a*)P for biotransformation after i.p. administration could be disposition in fat.

After intraperitoneal administration, Grimmer *et al.* (1988) and Jacob *et al.* (1989) found a total recovery of 6.2% for chrysene and of 3.1% for pyrene, respectively. These percentages were in the same order of magnitude as that found in the present study for B(*a*)P (2.2% mostly as 3-hydroxy-B(*a*)P). Egestadt *et al.* (1987) found 25% of the radioactivity of a dose of 22 μg ^{14}C-B(*a*)P to be extractable from feces. However, they used a combination of extraction with different solvents (80% ethanol, ethanol, hexane) and solid sorbents.

Sex differences

Males had a significantly higher excretion of mutagenic compounds as well as of 3-hydroxy-B(*a*)P in their urine than females. In feces, generally male rats excreted a higher percentage of B(*a*)P as metabolites than female rats. The differences between the sexes were not in the same order of magnitude for all metabolites. In vitro sex differences in biotransformation of PAH are described in literature. The formation of 3-hydroxy-B(*a*)P is regarded as indicative for total B(*a*)P metabolism by several authors (Gurtoo & Parker, 1977). Quantitative differences in the

formation of 3-hydroxy-B(*a*)P were confirmed in this study but this was not representative for other metabolites. It is likely that several isozymes of P450 are involved in the B(*a*)P metabolism: sex specific as well as non-sex specific.

Ethanol treatment

In the four groups investigated (female i.p., male i.p., female p.o., male p.o.) one particular effect of ethanol treatment was observed in the urine of female rats that received an i.p. dose of B(*a*)P. In this group, the ethanol treatment resulted in a statistically significant reduction of B(*a*)P metabolites in urine. Also, after p.o. and i.p. administration of B(*a*)P, 3-hydroxy-B(*a*)P was significantly lower after ethanol treatment in feces of males and females. Consequently, only a quantitative effect could be observed in both sexes. A reduced amount of metabolites in excretion products after ethanol treatment could be due to changes in absorption, biotransformation or excretion. Since the decrease in metabolite excretion in feces was found both after i.p. and p.o. administration of B(*a*)P, an ethanol treatment effect on the absorption of B(*a*)P through the gut seems unlikely.

There are several in vitro studies concerning the effect of ethanol treatment on biotransformation of B(*a*)P. Sato *et al.* (1986) measured a decrease, however not statistically significant, in B(*a*)P metabolism in rat liver microsomes of male Wistar rats. Murphy and Hecht also found a decrease in hepatic B(*a*)P metabolism in hamsters (Murphy & Hecht, 1986). In other studies, an increase in in vitro B(*a*)P metabolism was reported in liver and/or intestine (Seitz *et al.*, 1978; Seitz *et al.*, 1981)

Oxygenation by the monooxygenase linked P450 isozyme complex is considered as a first step in the biotransformation of B(*a*)P. Ethanol is known to act as an inducer of the monooxygenase linked cytochrome P450 (Rubin & Lieber, 1968; Onishi & Lieber, 1977; Koop & Coon, 1986). B(*a*)P is a compound activated by this enzyme system. The P450-isozyme that has the greatest affinity for B(*a*)P (IA1, characterized by Ethoxyresorufin O-deethylase [EROD] activity), however, is only slightly induced by ethanol treatment (Winston *et al.*, 1990). The isozyme which is typically induced by ethanol treatment, IIE1, is characterized by aniline-4-hydroxylase activity and has a low affinity for B(*a*)P (Koop & Coon, 1986). The reduction in the formation of some metabolites after ethanol treatment in the present analysis could be explained by a possible down regulating effect of ethanol on the constitutive isozymes that metabolize B(*a*)P (Wortelboer *et al.*, 1991; van de Wiel *et al.*, 1992).

The arene oxides that are formed from B(*a*)P can be further metabolized by glutathione S-transferases. The phenols, quinones and dihydrodiols of B(*a*)P may be converted by cytosolic sulfotransferases and by UDP glucuronyltransferases (Thakker *et al.*, 1985) Chronic ethanol administration enhances the hepatic glutathione content but does not affect glutathione S-transferase activity (Muñoz *et al.*, 1987; Yang & Carlson, 1991). Acute administration of ethanol shows

inhibition of glutathione synthesis and a decrease of glutathione S-transferase activity (Gonzalez *et al*, 1987) This could cause a decreased excretion of metabolites, but it is not likely to be selective for certain metabolites.

In conclusion: In *in vivo* studies we have observed clear differences in the amounts of benzo(*a*)pyrene metabolites in urine and feces between the sexes and between orally or intraperitoneally treated rats Also, an influence of ethanol treatment was found, but only with respect to the amounts of 3-hydroxy-B(*a*)P. This effect was more pronounced in the male rats. Whether the differences that were detected after ethanol treatment were due to biotransformational effects or effects on excretion mechanisms or other pharmacokinetic/pharmacodynamic parameters remains to be determined.

REFERENCES

Aitio A Different elimination and effect on mixed function oxidase of 20-methylcholanthrene after intragastric and intraperitoneal administration Res Com Chem Path Pharm, 9(4) (1974) 701-710

Bos, R B, R M E Brouns, R van Doorn, J L G Theuws, and P Th Henderson, The appearance of mutagens in urine of rats after the administration of benzidine and some other aromatic amines Toxicology, 16 (1980) 113-122

Bowes, S G and A G Renwick, The intestinal metabolism and DNA binding of benzo(*a*)pyrene in guinea-pigs fed normal, high-fat and high-cholesterol diets Xenobiotica, 16(6) (1986) 543-553

Chipman, J K, P C Hirom, G S Frost, and P Millburn, The biliary excretion and enterohepatic circulation of benzo(*a*)pyrene and its metabolites in the rat Biochem Pharmacol, 30(9) (1981) 937-944

Egestad, P, Pettersson, J Sjövall, J Rafter, K Hyvönen and J-A Gustafsson, Studies on the chromatographic fractionation of metabolites of benzo(*a*)pyrene in feces and urine from germfree and conventional rats Biomed Chrom, 20(3) (1987) 120-134

Foth, H, R Kahl, and G F Kahl, Pharmacokinetics of low doses of benzo(*a*)pyrene in the rat Food Chem Toxicol, 26 (1988) 45-51

Fournier, P E and Thomas, G The impact of life style factors and consumption of pharmaceutical drugs on toxicokinetics, in W R F Notten, R F M Herber, W J Hunter, A C Monster, R L Zielhuis (Eds), Health surveillance of individual workers exposed to chemical agents, Springer-Verlag, Berlin, 1986, p 22-31

Gonzalez, J, M E Muñoz, M I Martin, P S Collado, J Fermoso, and A Esteller, Influence of acute ethanol administration on hepatic glutathione metabolism in the rat Alcohol, 5 (1987) 103-106

Grimmer, G, H Brune, G Dettbarn, U Heinrich, J Jacob, E Mohtashamipur, K Norrpoth, F Pott, and R Wenzel-Hartung, Urinary and faecal excretion of chrysene and 1-OH-chrysene metabolites by rats after oral, intraperitoneal, intratracheal or intrapulmonary application Arch Toxicol, 62 (1988) 401-405

Gurtoo, H L and N Bejba Parker, Sex-dependent regulation of benzo(*a*)pyrene and zoxazolamine metabolism in rat tissues Drug Metab Disp, 5 (1977) 474-481

IARC, Monographs on the evaluation of carcinogenic risks to humans Alcohol Drinking, IARC, Lyon, France, 1988, vol 44

Jacob, J and H Brune, Urinary and faecal excretion of pyrene and hydroxypyrene by rats after oral, intraperitoneal, intratracheal or intrapulmonary application Cancer Lett , 46 (1989) 15-20

Johansson, I , G Ekström, B Scholte, D Puzycki, H Jörnvall, and M Ingelman-Sundberg, Ethanol-, fasting- and aceton-inducible cytochromes P-450 in rat liver regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies Biochemistry, 27 (1988) 1925-1934

Jongeneelen, F J , C M Leydekkers, and P Th Henderson, Urinary excretion of 3-hydroxy-benzo(*a*)pyrene after percutaneous penetration and oral absorption of benzo(*a*)pyrene in rats Cancer Lett , 25 (1984) 195-201

Kawamura, Y , T Unemura, M Wakana, T Kaneko, S Uchiyama, and Y Saito, Distribution, excretion and lymphatic absorption of benzo(*a*)pyrene administered orally in oil solution and aquous suspension forms J Food Hyg Soc Japan, 29(1) (1988) 26-31

Koop, D R and M J Coon, Ethanol oxidation and toxicity role of alcohol P-450 oxygenase Alcoholism Clin Exp Res , 10 suppl (1986)

Kotin, P , H L Falk, R Busser, Distribution, retention, and elimination of ^{14}C-3,4-benzpyrene after administration to mice and rats J Nat Cancer Inst , 23(3) (1959) 541-555

Maron, D M and B N Ames, Revised methods for the Salmonella mutagenicity test Mutat Res , 113 (1983) 173-215

Muñoz, M E , M I Martin, J Fermoso, J Gonzalez, and A Esteller, Effect of chronic ethanol feeding on glutathione and glutathione-related enzyme activities in rat liver Drug Alcohol Depend , 20 (1987) 221-226

Murphy, S E and S S Hecht, Effects of chronic ethanol consumption on benzo(*a*)pyrene metabolism and glutathion-S-transferase activities in Syrian Golden Hamster cheek pouch and liver Cancer Res , 46 (1986) 141-146

Ohnishi, K and C S Lieber, Reconstitution of the microsomal ethanol-oxidizing system J Biol Chem , 252 (1977) 7124-7131

Prasad, J S, D L Crankshaw, R R Erickson, C E Elliott, A D Husby, and J L Holtzman, Studies on the effect of chronic consumption of moderate amounts of ethanol on male rat hepatic microsomal drug metabolizing activity Biochem Pharmacol , 34(19) (1985) 3427-3431

Rubin, E and C S Lieber, Hepatic microsomal enzymes in man and rat Induction and inhibition by ethanol Science, 162 (1968) 691

Sato, A , I Yonekura, M Asakawa, H Nakahara, T Nakajima, S Ohta, T Shirai, and N Ito, Augmentation of ethanol-induced enhancement of dimethylnitrosamine and diethylnitrosamine metabolism by lowered carbohydrate intake Jpn J Cancer Res , (Gann) 77 (1986) 125-130

Seitz, H K , A J Garro, and C S Lieber, Effect of chronic ethanol ingestion on intestinal metabolism and mutagenicity of benzo(*a*)pyrene Biochem Biophys Res Comm , 85 (1978) 1061-1066

Seitz, H K , A J Garro, and C S Lieber, Sex dependent effect of chronic ethanol consumption in rats on hepatic microsome mediated mutagenicity of benzo(*a*)pyrene Cancer Lett , 13 (1981) 97-102

Smith, B R and J R Bend, Metabolism and excretion of benzo(*a*)pyrene 4,5-oxide by the isolated perfused rat liver Cancer Res , 39 (1979)a 2051-2056

Takahashi, G , H Shah, and S Weinhouse, Metabolism of [^{3}H]-3-methylcholanthrene in the perfused rat liver Cancer Res , 37 (1977) 369-375

Thakker, D R., H Yagi, W Levin, A W Wood, A H Conney, and D M Jerina, Polycyclic Aromatic Hydrocarbons Metabolic Activation to ultimate carcinogens, in M W Anders (Ed), Bioactivation of foreign compounds, Academic Press Inc ,New York, 1985, pp 172-242

Uziel, M and R Haglund, Persistence of benzo(*a*)pyrene and 7,8-dihydro-7,8-dihydroxy- benzo(*a*)pyrene in Fischer 344 rats, time distribution of total metabolites in blood, urine and feces Carcinogenisis, 9 (1988) 233-238

van de Wiel, J A G, C M P Duijf, J C L M Pertijs, J H J Copius Peereboom-Stegeman, and R P Bos, Growth and liver morphology after long-term ethanol consumption of rats Lab Anim, 24 (1990) 265-272

van de Wiel, J A G, M Meuwissen, H Kooy, P H S Fijneman, J Noordhoek and R P Bos, Influence of long-term ethanol treatment on *in vitro* biotransformation of benzo(*a*)pyrene in microsomes of the liver, lung and small intestine from male and female rats Biochem Pharmacol, 44 (1992) 1977-1984

Willems, M J and W K de Raat, Mutagenicity of urine and feces after treatment of rats with known genotoxins Carcinogenesis, 6 (1985) 967-976

Winston, G W, S Narayan, and P L Bounds, Profiles of ethanol-induced microsomal alkoxyresorufin(alkoxyphenoxazone)O-dealkylation comparison with phenobarbital- and aroclor 1254-induced systems Alcohol Alcoholism,25(6) (1990) 667-672

Wortelboer, H M, C A de Kruif, A A J van Iersel, H E Falke, J Noordhoek and B J Blaauboer, Acid reaction products of indole-3-carbinol and their effects on cytochrome P450 and phase II enzymes in rat and monkey hepatocytes, in H M Wortelboer, Primary hepatocyte cultures as a model system for the determination of induction of biotransformation enzymes, Utrecht, 1991

Yang, C M, and G P Carlson, Effects of ethanol on glutathione conjugation in rat liver and lung Biochem Pharmacol, 41 (1991) 923-929

CHAPTER IV

INFLUENCE OF LONG-TERM ETHANOL TREATMENT ON *IN VITRO* BIOTRANSFORMATION OF BENZO(*A*)PYRENE IN MICROSOMES OF THE LIVER, LUNG AND SMALL INTESTINE FROM MALE AND FEMALE RATS.

Jeanine A G van de Wiel, Marly Meuwissen, Heimen Kooy,
Peter H S Fijneman, Jan Noordhoek, Rob P. Bos

Department of Toxicology, Faculty of Medical Sciences,
University of Nijmegen, P O Box 9101,
6500 HB Nijmegen
The Netherlands

Biochemical Pharmacology 1992;44(10):1977-1984

ABSTRACT

The influence of long-term ethanol exposure of rats on microsomal biotransformation of benzo(*a*)pyrene [B(*a*)P] was studied. Male and female Wistar rats received an increasing amount of ethanol in their drinking water: percentages rose to 15% (w/v) in 3 weeks. The ethanol content was kept at a concentration of 15% for another 3 weeks. Liver, lungs and intestinal epithelial cells of the rats were then isolated and microsomal fractions prepared. In all organs, the metabolite most formed was 3-hydroxy-B(*a*)P. In liver, males showed significantly higher B(*a*)P hydroxylase activity than females. On the basis of experiments using monoclonal antibodies, a significant part of the B(*a*)P biotransformation in male rat liver microsomes can be attributed to the male specific P4502C11. In lung and intestine there were no significant differences between the sexes. In liver, ethanol treatment significantly decreased the microsomal formation of phenolic metabolites. In microsomes of intestinal epithelial cells ethanol treatment enhanced the formation of phenols and diols. In conlusion: in rats, ethanol consumption in moderate amounts leads to an alteration in the microsomal biotransformation of B(*a*)P. Effects are most prominent in liver, where the formation of phenols is significantly decreased.

INTRODUCTION

The impact of life style factors on toxicokinetics of foreign compounds is clearly recognized. The main, non professional, life style associated parameters are use of alcohol, smoking, drug and dietary habits (Fournier & Thomas, 1986). Interactions between alcohol intake and toxicokinetics of polycyclic aromatic hydrocarbons (PAHs) are of interest because PAHs are ubiquitous environmental contaminants and components of cigarette smoke. The risk of cancer of the larynx, pharynx and oesophagus is found to be additive or multiplicative for people who smoke *and* drink alcohol (IARC, 1988).

Ethanol has a broad spectrum of direct toxic effects (Lieber, 1984). Indirectly it acts as an inducer of the monooxygenase-linked cytochrome P450 complex (Rubin & Lieber, 1968; Onishi & Lieber, 1977; Koop & Coon, 1986; Johansson *et al.*, 1988). Conceivably, the additive or multiplicative effect of drinking and smoking on carcinogenesis could be attributed to an enhanced biotransformation of B(*a*)P into its reactive intermediates by ethanol-induced P450 monooxygenases. Findings by Seitz *et al.* (1978, 1981) support this idea. They measured enhanced aryl hydrocarbon hydroxylase activity in microsomes of liver and small intestine of female rats and in intestinal microsomes of male rats that were on an alcohol-containing diet (Seitz *et al.*, 1978, 1981, 1981a). Also, enhanced biotransformation of PAHs in the cheek pouch epithelium (but not in the liver) of Syrian golden hamsters was measured after long term ethanol treatment

(Murphy & Hecht, 1986). However, other authors found a decrease or no change in the *in vitro* metabolism of B(*a*)P by rat and hamster liver microsomes (DeMarco & McCoy, 1984; Sato *et al.*, 1986). The specific ethanol inducible P450 isoenzym (P4502E1) demonstrated little affinity for PAHs (Koop & Coon, 1986).

We have recently described the *in vivo* toxicokinetics of B(*a*)P after long-term ethanol treatment (van de Wiel *et al.*, 1993). After one i.p. or p.o. dose of B(*a*)P given to rats chronically exposed to ethanol via their drinking water, the excretion of 3-hydroxy-B(*a*)P was significantly decreased. The effect of ethanol treatment on B(*a*)P metabolite excretion measured in vivo was attributed to changes in absorption, biotransformation and/or excretion.

This paper presents data on the effects of long-term ethanol treatment on the biotransformation of B(*a*)P. Microsomes of lung, liver and small intestine from male and female rats were used. This allowed for a comparison of possible sex-related differences of B(*a*)P biotranformation in the three organs. Finally, to determine which isoenzyme of P450 was most important in B(*a*)P biotransformation, liver microsomes were incubated with monoclonal antibodies (Mabs) to the P450 isoenzymes; 1A1/2, 2E1, 2B1/2 and 2C11/6 to measure the inhibition of B(*a*)P metabolite formation.

MATERIALS AND METHODS

Chemicals

Ethanol used for the treatment was Ethanol absolute GR, supplied by Merck (Darmstadt, Germany). Benzo(*a*)pyrene (CAS 50-32-8), was purchased from Sigma (St. Louis, MO, USA). B(*a*)P metabolites were obtained from the NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas city, MO, USA). All other chemicals were of analytical grade, obtained from local commercial sources and used without further purification.

Animals

Homebred Cpb:WU (Wistar) rats of both sexes were used. Initial weights varied between 100-120g (females) and 120-150g (males). The age of the rats was about 6 weeks at the beginning of the experiments and 12 weeks at the end. The animals were of Specified Pathogen Free (SPF) quality. Antibodies against the PVM virus and the Pin-worm *Syphacia muris* but no other pathogens were found. The animals were kept in Macrolon type 3 cages on sterilized softwood granules as bedding. Per cage, three animals were housed.

The animals were provided with RMH-TM pellets (Hope Farms B.V. Woerden, The Netherlands). The rats had free access to water with an increasing ethanol content reaching up to 15% w/v after 6 weeks. Details of the method of

alcohol exposure have been described previously (van de Wiel *et al.*, 1990). Room temperature was regulated (22 ± 2°C), relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 7.00 a.m. and 7.00 p.m..

Preparation of microsomes

Six weeks after the start of the alcohol treatment the rats were anaesthetized with pentobarbital and perfused with ice-cold saline via the ventricle of the heart until the liver and lungs were decoloured. The livers were excised, weighed, collected in ice-cold 0.25 M sucrose, sliced and homogenized in 3 volumes ice-cold 0.25 M sucrose, using a Potter-Elvehjem glass teflon homogenizer. The cell debris and nuclei were removed at a first centrifugal run at 600 *g* (4°C). Lungs were also removed, sliced and homogenized in 5 volumes ice-cold 1.15% KCl in 10 mM Tris-HCl/2 mM EDTA and 10% (v/v) glycerol (pH=7.4). An intestinal segment consisting of the first 60 cm distal to the pylorus was excised, perfused free of intestinal content with an ice-cold isotonic KCl solution containing 0.05 M Tris-HCl buffer (pH=7.8), and slit open. The upper villous layer of the mucosa was removed by scraping with the edge of a glass slide. Next it was suspended in 4 ml of Tris-KCl with glycerol (20% v/v) and heparin (3 U/ml). To this suspension, trypsin inhibitor (5 mg/g wet weight of small intestine) was added, according to Stohs *et al.* (1976).

From all tissue homogenates mitochondria were removed by centrifugation for 20 min at 9000 *g*. The floating fat layer was removed and the underlying supernatant fraction was decanted and homogenized. The post-mitochondrial fraction was centrifuged at 105000 *g* for 75 min at 4°C. For the liver, the pellet was resuspended in ice-cold 1.15% KCl in 10 mM Tris-HCl (pH=7.4). The final suspension corresponded to 1 g liver per ml suspension. The microsomal pellet of the lungs was resuspended in 10 mM Tris-HCl (pH=7.4), according to Boyd *et al.* (1978). The final suspension corresponded to 2 grams tissue per ml. The intestinal microsomes were resuspended to a concentration corresponding to 2 g/ml in 10 mM Tris-HCl containing 1.15% (w/w) KCl, pH=7.4.

Microsomes of all organs were distributed in small samples, frozen in liquid nitrogen and stored at -80°C until use.

Assays

Protein concentrations were determined according to Lowry *et al.* (1951) using bovine serum albumin as the standard. The reduced, CO-bound cytochrome P450 difference spectra were determined according to Omura & Sato (1964) with modifications according to Rutten *et al.* (1987).

The frozen microsomes were thawed quickly at 37°C just prior to use. Benzo(*a*)pyrene metabolism was assayed with modifications of the procedure of

Yang *et al.* (1975). The reaction mixture contained 1.9 ml 0.1 M Sörensen buffer with NADPH (0.72 mM) and $MgCl_2.6H_2O$ (3.75 mM), 60 µl microsomal suspension and 50 µl B(*a*)P in acetone (100 nmol/ml incubation mixture). After a preincubation of two minutes at 37°C and shaking at 110 rpm (Gyrotory Water Bath Shaker, New Brunswick Scientific Co, Edison N.J. USA), the incubation was started by adding 50 µl of B(*a*)P solution. After 10 (liver) or 20 (lung, intestine) minutes the incubation was stopped by the addition of 2 ml acetone. B(*a*)P and metabolites were extracted in 4 ml ethyl acetate. After 30 minutes 3 ml of the organic layer was removed and evaporated under nitrogen. The residue was dissolved in methanol to a volume of 1.7 ml for liver and 0.5 ml for intestine and lung. The samples were kept in vials at -20°C until HPLC analysis.

Mab inhibition

Monoclonal antibodies (MAbs) were produced at the US National Cancer Institute, Laboratory of Molecular Carcinogenesis using a modification of the method of Koehler and Milstein (1975). They were tested and characterized as described by Park *et al.* (1986, 1989) and Ko *et al.* (1987). In the present study four MAbs shown to be specific towards different cytochromes P450s were used as follows; clone 1-7-1 to MC-inducible P4501A1, which cross-reacted with P4501A2, clones 2-66-3 to PB-inducible P4502B1, which cross-reacted with P4502B2, clone 1-91-3 to ethanol-inducible P4502E1 and clone 1-68-11 to P4502C11, which cross-reacted with P4502C6 (Nakajima *et al.*, 1991). Antibody was added at concentrations of 0, 10, 50 and 100% of the microsomal protein in the incubation mixture. Control MAb (Hy-Hel) was used in the same amounts to determine any non-specific reaction. MAbs were added to microsomes and buffered at room temperature 30 min prior to starting the B(*a*)P metabolism assay at 37°C by adding NADPH and substrate.

Reversed-phase HPLC analysis and detection of B(a)P metabolites

Analyses were performed with a Spectra Physics HPLC (SP8800) equipped with two solvent pumps, a solvent programmer and an automatic sampler (SP8775). Using a sample loop, a 20 µl aliquot was injected onto a 150x4.6 mm ID Nucleosil C18 (10 µm) column. Column temperature was 40°C, flow 1.0 ml per min. The following solvent programme was 5 min 90% solvent A (60% aqua pure, 40% methanol), a linear gradient to 10% solvent A and 90% solvent B (100% methanol) in 40 min followed by 10 min 90% solvent B.

Fluorescence detection was performed with a Perkin Elmer LS-4 spectrofluorimeter. The programme for excitation and emission wavelengths during each run is given in Table 1. The retention times of B(*a*)P-9,10-dihydrodiol, B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol and B(*a*)P were 19, 26, 27 and 43 minutes respectively. The retention times of

9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P were 36.5 and 37.0 ± 0.5 min, respectively. Detection limits for 3-hydroxy-B(*a*)P, B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol, and B(*a*)P-9,10-dihydrodiol were 0.05 nmol/ml injected volume. For 9-hydroxy-B(*a*)P it was 0.01 nmol/ml. Quantification was done by measuring peak heights and comparing these with a standard range incubated and extracted as described for the microsomal incubation samples.

Time (min)	excitation (nm)	emission (nm)	metabolites detected
0	280	406	B(a)P-9,10-dihydrodiol
21	265	405	B(a)P-4,5-dihydrodiol/ B(a)P-7,8-dihydrodiol
31	265	430	3-hydroxy-B(a)P/9-hydroxy-B(a)P
41	296	407	B(a)P
58	C*	C	

Table 1 Program of Perkin Elmer LS-4 detector
*End of program, back to position at time=0

Statistical analysis

Results are presented as mean values ± standard errors of the mean. Statistical analysis was carried out using the SAS (Statistical Analysis System) package on a VAX6410 minicomputer. The procedure used was GLM (general linear model) for analysis of variance with unequal cells. A p-value <0.05 was considered to be of statistical significance.

With the analysis of variance procedure, it was possible to take more than two groups (of rats) and more than one variable (metabolites) into account. The procedure was always applied to four groups (male ethanol, male control, female ethanol, female control). The number of variables (metabolites) tested in one procedure was different for each organ. With the liver samples, each individual metabolite was tested for statistical difference between males and females and ethanol-treated and control animals. With lung and intestinal samples, all metabolites were used in one analysis because only few samples per group were obtained.

RESULTS

Liver

Mean levels of protein and P450 of microsomes from long-term ethanol treated male and female rats are shown in Table 2. The protein levels were significantly higher in ethanol-treated rats, in both males and females. The levels of P450 are significantly higher in males compared to females and in ethanol-treated rats compared to non-treated rats.

Rats	n	protein mg/ml ± sem	P450 nmol/mg prot ± sem
M 0% EtOH	6	15.6 ± 0.5	0.77 ± 0.03
M 15% EtOH	6	18.1 ± 0.9*	1 06 ± 0 06*
F 0% EtOH	6	13.5 ± 1.2	0.65 ± 0.02
F 15% EtOH	6	21.3 ± 0.7*	0.74 ± 0.02*

Table 2. Protein and P450 levels of liver microsomes from ethanol-treated and non-treated male (M) and female (F) rats
n=number of rats sem=standard error of the mean
* significant difference between ethanol-treated and non-treated rats $p<0$ 05

The rate of formation of B(*a*)P metabolites after incubation of B(*a*)P with liver microsomes is shown in Table 3. Quantitatively, the most prominent metabolite formed was 3-hydroxy-B(*a*)P: it amounted to 75% of all metabolites. In males, B(*a*)P-9,10-dihydrodiol was 10% of all metabolites and B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol and 9-hydroxy-B(*a*)P were each 5% of all metabolites measured. In microsomes of female rats 9-hydroxy-B(*a*)P was the second important metabolite formed (10% of all metabolites measured) and the diols each amounted to 5% of the metabolite total. Male rats showed significantly higher specific B(*a*)P hydroxylase activity than females either per mg protein or per nmol P450 ($p<0.001$).

Ethanol-treated rats showed a significantly decreased rate of formation of 3-hydroxy-B(*a*)P and 9-hydroxy-B(*a*)P per mg protein in comparison with non-treated rats. There is a curious sex-related difference in the mean diol levels. Female rats seem to have a higher diol formation rate after ethanol treatment. On the contrary, male rats have a lower diol formation rate after ethanol treatment. However, these differences were not statistically significant.

	0% EtOH		15% EtOH	
Metabolite	males	females	males	females
3-hydroxy-B(a)P*	601 ± 47	127 ± 9	425 ± 27*	98 ± 4
9-hydroxy-B(a)P*	23 ± 2	14 ± 1	20 ± 5*	5 ± 0
B(a)P-4,5-dihydrodiol	27 ± 3	5 ± 1	25 ± 4	11 ± 2
B(a)P-7,8-dihydrodiol	42 ± 3	7 ± 1	35 ± 1	8 ± 0
B(a)P-9,10-dihydrodiol**	94 ± 9	9 ± 1	73 ± 9	21 ± 2

Table 3. Rate of formation of B(*a*)P metabolites in liver microsomes of male and female rats
Values are pmol/mg protein/min, means ± SEM
N=6 sem=standard error of the mean
* significant difference between ethanol-treated and non-treated rats, ** significant interaction between ethanol treatment and sex p>0 05.

Lung

Mean levels of protein and P450 of microsomes from male and female rats after long term ethanol treatment are shown in Table 4. There were no significant differences in the protein levels nor in the P450 levels (per mg protein) between the sexes and between ethanol-treated and non-treated rats.

Rates of formation of B(*a*)P metabolites in in vitro experiments with lung microsomes are shown in Table 5. Only two samples could be measured in each group because of the very small amount of microsomes obtained from one animal. Both in males and females, 3-hydroxy-B(*a*)P comprised 70% of all metabolites measured and the diols 9% each. 9-Hydroxy-B(*a*)P was the least important metabolite, about 3% of all metabolites measured. With all metabolites taken together in the analysis of variance, no significant difference between males and females in the formation rate of B(*a*)P metabolites was found. No significant effect of ethanol treatment was observed either.

Rats	n	protein mg/ml ± sem	P450 nmol/mg prot ± sem
M 0% EtOH	2	9 9 ± 1 3	0 062 ± 0 01
M 15% EtOH	2	9 3 ± 1 4	0 124 ± 0 03
F 0% EtOH	2	9.7 ± 0 6	0 093 ± 0 08
F 15% EtOH	2	8 9 ± 0 9	0 080 ± 0 03

Table 4 Protein and P450 levels of lung microsomes n=number of samples Each sample consisted of pooled lung microsomes of 5-6 rats sem=standard error of the mean

	0% EtOH		15% EtOH	
Metabolite	male	female	male	female
3-hydroxy-B(a)P	3 55 ± 1 15	3 34 ± 0 16	2 89 ± 0 25	2 92 ± 0 02
9-hydroxy-B(a)P	0 22 ± 0 04	0 13 ± 0 01	0 13 ± 0 01	0 10 ± 0 00
B(a)P-4,5-dihydrodiol	0 39 ± 0 15	0 34 ± 0 02	0 33 ± 0 02	0 35 ± 0 06
B(a)P-7,8-dihydrodiol	0 51 ± 0 20	0 37 ± 0 07	0 47 ± 0 02	0 35 ± 0 00
B(a)P-9,10-dihydrodiol	0 45 ± 0 22	0 37 ± 0 16	0 47 ± 0 01	0 26 ± 0 07

Table 5 Rate of formation of B(*a*)P metabolites in lung microsomes of male and female rats
Number of samples=2 Each sample consisted of pooled lung microsomes of 5-6 rats Values are pmol/mg protein/min, means ± SEM
sem=standard error of the mean

Intestinal epithelium

Mean levels of protein and P450 of microsomes from male and female rats after long-term ethanol treatment are shown in Table 6. Protein Levels were significantly decreased in ethanol-treated compared to non-treated rats. There were no statistically significant differences between the sexes and between ethanol-treated and non-treated rats with respect to the levels of P450 in the microsomes of small intestine.

Both in males and females, 3-hydroxy-B(*a*)P comprised about 85% of all metabolites formed, while the other metabolites amounted to only a few percent each Taking all B(*a*)P metabolites into account, B(*a*)P hydroxylation was significantly faster in males than in females ($p=0.016$) and they were significantly enhanced by ethanol treatment ($p=0.035$). These results are presented in Table 7.

Rats	n	Protein mg/ml ± sem	P450 nmol/mg prot ± sem
M 0% EtOH	4	16 7 ± 0 9	0 068 ± 0 01
M 15% EtOH	4	10 1 ± 2 6*	0 085 ± 0 02
F 0% EtOH	4	11 9 ± 0 5	0 070 ± 0 01
F 15% EtOH	4	10 8 ± 0 8*	0 053 ± 0 01

Table 6 Protein and P450 levels of microsomes of small intestine
n=amount of samples Each sample consisted of pooled intestinal microsomes of 2-3 rats
sem=standard error of the mean
* significant difference between ethanol-treated and non-treated rats p>0 05

	0% EtOH		15% EtOH	
Metabolite	male	female	male	female
3-hydroxy-B(a)P	8 39 ± 1 27	6 70 ± 1 08	12 47 ± 1 30	8 00 ± 2 20
9-hydroxy-B(a)P	0 95 ± 0 16	0 91 ± 0 12	1 48 ± 0 12	1 06 ± 0 17
B(a)P-4,5-dihydrodiol	0 49 ± 0 14	0 45 ± 0 09	0 70 ± 0 15	0 61 ± 0 10
B(a)P-7,8-dihydrodiol	0 42 ± 0 07	0 20 ± 0 05	0 44 ± 0 23	0 16 ± 0 09
B(a)P-9,10-dihydrodiol	0 09 ± 0 05	0 03 ± 0 03	0 14 ± 0 08	0 03 ± 0 03

Table 7 Rate of formation of B(*a*)P metabolites in microsomes of small intestine of male and female rats
Values are pmol/mg protein/min, means ± SEM
Number of samples=4 Each sample consisted of pooled intestinal microsomes of 2-3 rats sem=standard error of the mean
Significant difference between ethanol-treated and non-treated rats $p < 0\ 05$ for all metabolites

Inhibition of B(a)P metabolism by Monoclonal Antibodies

In Table 8, the inhibition of B(*a*)P metabolite formation by MAb 1-68-11 (anti-P4502C11) is shown. The formation of all B(*a*)P metabolites was significantly inhibited. MAbs 1-7-1 (anti-P4501A1), 1-91-3 (anti-P4502E1) and 2-66-3 (anti-P4502B1) did not inhibit the formation of B(*a*)P metabolites or only to a minor extent (data not shown). The residual activity of B(*a*)P hydroxylase after inhibition with MAb 1-68-11 differed considerably, depending on the metabolite measured. The formation of B(*a*)P-9.10-dihydrodiol was reduced to 17% of the initial value, whereas the formation of B(*a*)P-4,5-dihydrodiol was reduced only to 79%. For 3-hydroxy-B(*a*)P, B(*a*)P-4,5-dihydrodiol and

B(*a*)P-7,8-dihydrodiol there was a significant difference in residual activity between ethanol-treated and non-treated rats when the amount of MAb protein in the incubation mixture was 50% of the amount of microsomal protein. The remaining activity was significantly higher in ethanol-treated rats.

Treatment	Metabolites*	1-68-11(P450IIC11/6) (nmol/mg protein/min) 0%	50%	100%
0% EtOH	3-hydroxy-B(*a*)P	1 460 ± 0 203 (100)	0 435 ± 0 142 (46)	0 435 ± 0 142 (31)
	9-hydroxy-B(*a*)P	0 013 ± 0 002 (100)	0 006 ± 0 (42)	0 006 ± 0 (43)
	B(*a*)P-4,5-dihydrodiol	0 024 ± 0 001 (100)	0 018 ± 0 002 (75)	0 018 ± 0 001 (75)
	B(*a*)P-7,8-dihydrodiol	0 052 ± 0 004 (100)	0 025 ± 0 002 (49)	0 021 ± 0 002 (40)
	B(*a*)P-9,10-dihydrodiol	0 128 ± 0 018 (100)	0 022 ± 0 001 (17)	0 022 ± 0 (17)
15% EtOH	3-hydroxy-B(*a*)P	1 240 ± 0 022 (100)	0 627 ± 0 011* (84)	0 472 ± 0 207 (38)
	9-hydroxy-B(*a*)P	0 015 ± 0 001 (100)	0 008 ± 0 (54)	0 007 ± 0 001 (45)
	B(*a*)P-4,5-dihydrodiol	0 026 ± 0 003 (100)	0 026 ± 0 * (87)	0 020 ± 0 006 (79)
	B(*a*)P-7,8-dihydrodiol	0 054 ± 0 001 (100)	0 042 ± 0 001* (67)	0 023 ± 0 003 (42)
	B(*a*)P-9,10-dihydrodiol	0 132 ± 0 007 (100)	0 034 ± 0 003 (26)	0 031 ± 0 006 (23)

Table 8 Inhibition of B(*a*)P metabolite formation by MAb in microsomes of male Wistar rat liver
Values are means of two rat liver samples ± SEM
In parenthesis the percentage of remaining activity after incubation with the antibody is shown, expressed as (activity with MAb/activity with Hy-Hel) x 100
* Significantly different from non-ethanol pretreated rats $p>0\ 05$

DISCUSSION

Microsomes from liver, lung and small intestine from male and female rats were used to estimate the effect of ethanol consumption on the biotransformation of B(*a*)P.

Quantitatively, the most important metabolite formed in all organs, 3-hydroxy-B(*a*)P, reached up to 70-85% of all metabolites measured. Other authors quoted only 36% in liver microsomes from Sprague Dawley rats (Yang *et al.*,1975) and 10% in intestinal microsomes from Wistar rats (Gower & Wills, 1986). In earlier studies *in vivo*, we found 95% of all excreted metabolites in urine and feces to be 3-hydroxy-B(*a*)P (van de Wiel *et al.*, 1993). This percentage is in agreement with our present results *in vitro*. The second most important metabolite

was different in liver, lung and small intestine. In intestinal microsomes, 9-hydroxy-B(*a*)P was the second important metabolite. In lung microsomes it was B(*a*)P-7,8-dihydrodiol and in liver microsomes B(*a*)P-9,10-dihydrodiol.

There were large differences in microsomal activity between the three organs. In liver B(*a*)P hydroxylase activity was about 600 pmol/mg protein per minute. In lung, we found 3 pmol/mg protein per minute. In the small intestine it was 10 pmol/mg protein per minute. The different rates of metabolic activity found in these organs are in agreement with the results of other investigators (Benford & Bridges, 1983).

Benford and Bridges (1983) found almost equal values in liver and small intestine of male Wistar rats. Only in lung microsomes they found higher activity than we did, about the same as in the small intestine.

In vivo we measured sex differences with respect to 3-hydroxy-B(*a*)P excretion in urine and feces; the excretion in males was significantly higher than in female rats. This in fact was reproduced in the present experiments in vitro, especially in liver and intestinal microsomes. 9-Hydroxy-B(*a*)P showed no significant sex related difference in vivo, and no sex related difference in liver microsomes either. In the organs investigated in this study, males generally showed higher activity than females. Blanck *et al.* (1986) showed that oxidative pathways of B(*a*)P biotransformation are controlled by pituitary hormones in a way similar to the rat-liver metabolism of steroids. The results of Yamazoe *et al.* (1986) indicate that the concentration of P450-male (P4502C11) in liver microsomes correlates with B(*a*)P hydroxylation activity and that both could be regulated by the serum growth hormone level. Gurtoo and Parker (1977) showed a sex related difference with a factor 6 for AHH activity in liver microsomes of Wistar rats and no sex related difference in lung microsomes. We found a sex related difference, with a factor of 2-5 for formation of diols and phenols in liver microsomes, no difference in lung microsomes and a small difference in microsomes of the small intestine. Gurtoo and Parker (1977) suggest an organ specificity for sex dependent regulation of microsomal mixed function oxydase activity. Direct evidence concerning the participation of the male specific isozyme in B(*a*)P biotransformation was provided by Todorovic *et al.* (1991). These authors inhibited microsomal B(*a*)P metabolising activity using an antibody to P4502C11, a male specific isozyme. Using the same antibody we were able to inhibit 21-83% of the microsomal B(*a*)P metabolism, depending on the metabolite measured. Like Todorovic *et al.* (1991), we also measured the greatest inhibitory effect of Mab 1-68-11 on formation of B(*a*)P-9,10-dihydrodiol (83%) and the least inhibitory effect on formation of B(*a*)P-4,5-dihydrodiol (25%). We also found considerable inhibition in the formation of the phenolic B(*a*)P metabolites, 54 and 58% for 3-hydroxy-B(*a*)P and 9-hydroxy-B(*a*)P, respectively. Todorovic *et al.* measured only 25%. In Sprague Dawley rats the amount of sex specific enzymes as constitutive forms of P450 is 20-25% of total P450 (Kamataki *et al.*, 1983,

1986) Probably, P4502C11 plays an important role in B(*a*)P biotransformation in uninduced, as well as in ethanol-treated rats This is largely different from rats that are treated with the 'classic' inducers 3-methylcholanthrene (3-MC) and Aroclor 1254 3-MC induces P4501A1 that largely metabolises B(*a*)P

The effect of ethanol treatment was different in the three organs In liver the B(*a*)P biotransformation was significantly decreased for the formation of the phenols 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P. For other P450 inducers like phenobarbital, 3-methylcholanthrene or PCB's it is known that they leave the sex specific P450 isozymes unaffected or depress them (Wortelboer *et al*, 1991, Wiebel & Gelboin, 1975) This also seems to be true for ethanol When using antibodies to P4502C11/6, the percentage remaining activity of B(*a*)P metabolite formation was significantly higher in ethanol-treated, compared to control rats This confirmed the down-regulating influence of ethanol on sex specific P450 isozyme activity and consequently on B(*a*)P biotransformation In the lung also, a decrease in B(*a*)P metabolism was observed, but not a significant one In small intestine, a significant increase in the metabolism of B(*a*)P was seen An increase in B(*a*)P biotransformation in small intestine was also decribed by Seitz *et al* (1978) For the human situation, combined exposure to alcohol and PAH often occurs It should be realized that ethanol induction could have implications with respect to an altered biologically effective dose of mutagenic/ carcinogenic PAH metabolites in several organs

In conclusion, we found differences between the sexes in the *in vitro* biotransformation of B(*a*)P in agreement with our studies *in vivo* Males in general have a greater metabolising capacity for B(*a*)P than females The influence of ethanol treatment on B(*a*)P biotransformation is different for each organ, but in liver the formation of phenolic metabolites is significantly decreased Most probably, in the liver a sex specific isozyme of P450 (P4502C11) is involved in the biotransformation of B(*a*)P This isozyme is depressed by ethanol treatment

REFERENCES

Benford DJ and Bridges JW, Tissue and sex differences in the activation of aromatic hydrocarbon hydroxylases in rats *Biochem Pharmacol* **32** 309-313, 1983

Blanck A, Aström A, Hansson T, De Pierre JW and Gustafsson J-Å, Pituitary regulation of cytochrome P450-mediated metabolism of steroids and xenobiotics in rat liver microsomes *Carcinogenesis* **7** 575-582, 1986

Boyd MR, Burka LT, Wilson BJ and Sasame HA, in vitro studies on the metabolic activation of the pulmonary toxin, 4-ipomeanol, by rat lung and liver microsomes *Jour Pharm* **207** 677-686, 1978

DeMarco GJ and McCoy GD, Comparison of effect of chronic ethanol consumption by Syrian golden hamsters on liver metabolism and mutagenicity of benzo(a)pyrene and N-nitrosopyrrolidine *Proc Am Assoc Cancer Res* **25** 320, 1984

Fournier PE and Thomas G, The impact of life style factors and consumption of pharmaceutical drugs on toxicokinetics In Health surveillance of individual workers exposed to chemical (eds Notten WRF, Herber RFM, Hunter WJ, Monster AC, Zielhuis RL) pp 22-31 Springer-Verlag, Berlin, 1986

Gower JD and Wills ED, The dependence of the rate of BP metabolism in the rat small intestinal mucosa on the composition of the dietary fat *Nutrition Cancer* **8** 151-161, 1986

Gurtoo HL and Bejba Parker N, Sex-dependent regulation of benzo(a)pyrene and zoxazolamine metabolism in rat tissues *Drug Metab Disp* **8** 474-481, 1977

IARC Alcohol drinking. IARC Monographs on the evaluation of carcinogenic risks to humans Alcohol Drinking vol 44, IARC, Lyon, France, 1988

Johansson I, Ekström G, Scholte B, Puzycki D, Jornvall H and Ingelman-Sundberg M, Ethanol-, fasting- and aceton-inducible cytochromes P-450 in rat liver regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies *Biochemistry* **27** 1925-1934, 1988

Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato, R, Sex difference of cytochrome P450 in the rat, Purification, Characterization and Quantitation of constitutive forms of cytochrome P450 from liver microsomes of male and female rats *Arch Biochem Biophys* **225** 758-770, 1983

Kamataki T, Maeda K, Shimada M and Kato R, Effects of phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls on sex-specific forms of cytochrome P450 in liver microsomes of rats *J Biochem* **99** 841-845, 1986

Ko I-Y, Park SS, Song BJ, Patten C, Tan Y, Hah YC, Yang CS and Gelboin HV, Monoclonal antibodies to ethanol-induced rat liver cytochrome P450 that metabolizes aniline and nitrosamines *Cancer Res* **47** 3101-3109, 1987

Koehler G and Milstein C, Continuous cultures of fused cells secreting antibody of predefined specificity *Nature* **256** 495-497, 1975

Koop DR and Coon MJ, Ethanol oxidation and toxicity role of alcohol P-450 oxygenase *Alcoholism Clin Exp Res* **10** suppl, 1986

Lieber CS, Metabolism and metabolic effects of alcohol *Med Clin N Am* **68** 3-31, 1984

Lowry OH,Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent *J Biol Chem* **193** 265-275, 1951

Murphy SE and Hecht SS, Effects of chronic ethanol consumption on benzo(a)pyrene metabolism and glutathion-S-transferase activities in Syrian Golden Hamster cheek pouch and liver *Cancer Res* **46** 141-146, 1986

Nakajima T, Wang RS, Elovaara E, Park SS, Gelboin HV, Hietanen E, Vainio H, Monoclonal antibody-directed characterization of cytochrome P450 isozymes responsible for toluene metabolism in rat liver *Biochem Pharmacol* **41** 395-404, 1991

Omura R and Sato R, The carbon monoxide-binding pigment of liver microsomes *J Biol Chem* **239** 2370-2378, 1964

Onishi K and Lieber CS, Reconstitution of the microsomal ethanol-oxidizing system *J Biol Chem* **252** 7124-7131, 1977

Park SS, Ko I, Patten C, Yang CS, Gelboin HV, Monoclonal antibodies to ethanol induced cytochrome P450 that inhibit aniline and nitrosamine metabolism *Biochem Pharmacol* **35** 2855-2858, 1986

Park SS, Waxman DJ, Lapenson DP, Schenkman JB, Gelboin HV, Monoclonal antibodies to rat liver cytochrome P4502c/RLM5 that regiospecifically inhibit steroid metabolism *Biochem Pharmacol* **38** 3067-3074, 1989

Rubin E and Lieber CS, Hepatic microsomal enzymes in man and rat Induction and inhibition by ethanol *Science* **162** 691, 1968

Rutten AJJL, Falke HE, Catsburg JF, Topp R, Blaauboer BJ, van Holsteijn I, Doorn L and van Leeuwen FXR, Interlaboratory comparison of total cytochrome P450 and protein determinations in rat liver microsomes *Arch Toxicol* **61** 27-33, 1987

Sato A, Yonekura I, Asakawa M, Nakahara H, Nakajima T, Ohta S, Shirai T and Ito N, Augmentation of ethanol-induced enhancement of dimethylnitrosamine and diethylnitrosamine metabolism by lowered carbohydrate intake *Jpn J Cancer Res (Gann)* **77** 125-130, 1986

Seitz HK, Garro AJ and Lieber CS, Effect of chronic ethanol ingestion on intestinal metabolism and mutagenicity of benzo(a)pyrene *Biochem Biophys Res Comm* **85** 1061-1066, 1978

Seitz HK, Garro AJ and Lieber CS, Sex dependent effect of chronic ethanol consumption in rats on hepatic microsome mediated mutagenicity of benzo(a)pyrene *Cancer lett* **13** 97-102, 1981a

Seitz HK, Garro AJ and Lieber CS, Enhanced pulmonary and intestinal activation of procarcinogens and mutagens after chronic ethanol consumption in the rat *Europ Jour Clin Inves* **11** 33-38, 1981

Stohs SJ, Grafström RC, Burke MD, Moldéus PW and Orrenius SG, The isolation of rat intestinal microsomes with stable cytochrome P450 and their metabolism of ben-zo()pyrene *Arch Biochem Biophys* **177** 105-116, 1976

Todorovic R, Devanesan PD, Cavalieri EL, Rogan EG, Park SS and Gelboin HV, A monoclonal antibody to rat liver cytochrome P450IIC11 strongly and regiospecifically inhibits constitutive benzo(a)pyrene metabolism and DNA binding *Mol Carc* **4** 308-314, 1991

Wiebel FJ and Gelboin HV, Aryl hydrocarbon (benzo[a]pyrene) hydroxylases in liver from rats of different age, sex and nutritional status (Distinction of two types by 7,8-benzoflavon) *Biochem Pharmacol* **24** 1511-1515, 1975

van de Wiel JAG, Excretion of benzo(*a*)pyrene and metabolites in urine and feces of rats Influence of route of administration, sex and long-term ethanol treatment *Toxicology* **80** 103-115, 1993

van de Wiel JAG, Duijf CMP, Pertijs JCLM, Copius Peereboom-Stegeman,JHJ and Bos, RP, Growth and liver morphology after long-term ethanol consumption of rats *Lab Anim* **24** 265-272, 1990

Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J en Blaau- boer BJ, Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures The effects of model inducers on apoproteins and biotransformation activities *Biochem Pharmacol* **42** 381-390, 1991

Yamazoe Y, Shimada M, Kamataki T, Kato R, Effects of hypophysectomy and growth hormone treatment on sex-specific forms of cytochrome P450 in relation to drug and steroid metabolisms in rat liver microsomes *Jap Jour Pharm* **42** 371-382, 1986

Yang SK, Selkirk JK, Plotkin EV and Gelboin HV, Kinetic analysis of the metabo-lism of benzo()pyrene to phenols, dihydrodiols, and Quinones by high-pressure chromatography compared to analysis by aryl hydrocqrbon hydroxylase assay, and the effect of enzyme induction *Cancer Res* **35** 3642-3650, 1975

CHAPTER V

INFLUENCE OF LONG-TERM ETHANOL TREATMENT ON RAT LIVER BIOTRANSFORMATION ENZYMES

J.A.G. van de Wiel*, P.H.S. Fijneman*, K.B. Teeuw*, B. van Ommen**, J. Noordhoek*, R.P. Bos*

* Department of Toxicology, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen The Netherlands

** Department of Biological Toxicology, TNO Toxicology and Nutrition Institute, Zeist, The Netherlands

Alcohol 1993, in press

ABSTRACT

The influence of long-term ethanol consumption of rats on liver enzymes that could be involved in the biotransformation of benzo(*a*)pyrene [B(*a*)P] has been studied. Male and female Wistar rats received an increasing amount of ethanol in their drinking water up to 15% (w/v) in 3 weeks. The ethanol content was kept at a concentration of 15% for another 3 weeks. One group of rats also received B(*a*)P in the last week of the ethanol treatment. Livers were isolated and microsomal and cytosolic fractions were prepared. In every enzyme measurement sex differences were observed. Long-term ethanol consumption induced P450, especially aniline 4-hydroxylase (P4502E1). However, testosterone 6ß-hydroxylase (P4503A2 and P4502C13) in males and testosterone 12ß-hydroxylase in females were decreased. The phase 2 enzymes glutathione S-transferase (subunit 1) and epoxide hydrolase were also decreased in their activity. Our results support the hypothesis that the effect of long-term ethanol consumption on B(*a*)P biotransformation as found in *in vivo* and *in vitro* studies, consisting of lowered formation of phenolic and diolic metabolites, is the result of a decrease of constitutive P450 isoenzymes.

INTRODUCTION

Ethanol acts as an inducer of the monooxygenase-linked cytochrome P450 complex (Johansson *et al.*, 1988; Koop & Coon, 1986; Lieber *et al.*, 1968; Onishi & Lieber, 1977). Therefore, it is conceivable that the cancer risk due to compounds that are bioactivated into electrophilic intermediates by monooxygenase activity can be increased (Lieber, 1990). An increased incidence of cancer has been found in alcoholics (Tuyns, 1990). Alcohol use and smoking leads to a more than additional cancer risk for organs of the upper intestinal and respiratory tract (Wynder *et al.*, 1957, 1961). Cigarette smoke contains polycyclic aromatic hydrocarbons (PAH), a widespread group of environmental contaminants. Some of them are mutagenic and carcinogenic such as benzo(*a*)pyrene [B(*a*)P]. This compound has to be activated by the monooxygenase system in order to become electrophilic.

Earlier, we studied the pharmacokinetics of this compound in rats after long-term ethanol treatment (van de Wiel *et al.*, 1993). *In vivo*, after one dose of B(*a*)P the excretion of phenolic metabolites was significantly decreased in urine and feces of ethanol-treated rats compared to controls. Although not significantly, the excretion of diols was also decreased. This was observed for both male and female rats. The decreased formation of these B(*a*)P metabolites was confirmed in an *in vitro* study using liver microsomal fractions (van de Wiel *et al.*, 1992). The results suggest that the effect of ethanol treatment on metabolite excretion levels measured in vivo is caused by changes in the biotransformation.

In the literature, data about effects of ethanol treatment on benzo(*a*)pyrene biotransformation seem to be conflicting. Benzo(*a*)pyrene hydroxylation has been reported to be increased in rat liver microsomal preparations after chronic ethanol feeding (Rubin & Lieber, 1968; Seitz *et al.*, 1978) but to be decreased in mouse liver microsomal preparations (Capel *et al.*, 1978).

Much heterogeneity exists among the hepatic monooxygenases. Ethanol induces specific forms of P450; P4502E1 and P4502B1 and P4502B2 (Johansson *et al.*, 1988). Purified rat P4502E1 has no significant activity towards benzo(*a*)pyrene (Ryan *et al.*, 1985). Benzo(*a*)pyrene is known to be a good substrate for P4501A1, but also for the sex-specific isoenzymes P4502C11 and P4503A2 (Kobliakov *et al.*, 1991). In the present study, the influence of long-term ethanol treatment on biotransformation capacity is monitored by measuring the metabolism of several substrates indicative for certain isoenzymes of P450.

We have used the same protocol of ethanol induction as applied in our *in vivo* and *in vitro* studies (van de Wiel, 1992, 1993). Male and female rats were used to observe possible sex differences. One group of rats was treated with ethanol and B(*a*)P simultaneously to study the effect of long term exposure to both inducers on the biotransformation of B(*a*)P.

Ethoxyresorufin O-deethylase is measured as an indicator enzyme for the activity of P4501A1. The increase in aniline hydroxylation is used as a marker for ethanol-dependent increases in P450 mediated metabolism (Koop & Coon, 1986). This represents the activity of the isoenzyme P4502E1 (Johansson *et al.*, 1988). Pentoxyresorufin O-deethylase (PROD) activity is used as a marker for P4502B1/P4502B2 isoenzymes. Testosterone metabolism can be correlated with the sex specific isoenzymes. In males 2α- and 16α-hydroxy-testosterone (OHT) activity corresponds with P4502C11, 6ß-OHT activity with P4503A2 and P4502C13 and 15α- and 7ß-OHT activity with P4502A2 (Kobliakov *et al.*, 1991). Besides P450 coupled biotransformation activities, glutathione S-transferase activity and subunit composition were studied because of the important role this enzyme plays in the detoxification of reactive B(*a*)P metabolites. For the same reason epoxide hydrolase was monitored.

METHODS

Chemicals

Ethanol used for the treatment was Ethanol absolute GR, supplied by Merck (Darmstadt, Germany). Resorufin was obtained from Aldrich (Brussels, Belgium). Ethoxyresorufin and pentoxyresorufin were from Boehringer (Mannheim, Germany). 1-Cloro-2,4-dinitrobenzene was from Sigma (St Louis, MO, USA) and styrene oxide was obtained from Fluka (Buchs, Switzerland). Testosterone, androstenedione, and the hydroxytestosterone (OHT) metabolites (2α-OHT,

7α-OHT, 16α-OHT, 6ß-OHT, 11ß-OHT, 16ß-OHT and 19-OHT) were a gift from Dr. Heleen Wortelboer, RITOX Institute, Utrecht, the Netherlands. All other chemicals were of analytical grade, obtained from local commercial sources and used without further purification.

Animals

Homebred Cpb:WU (Wistar) rats of both sexes were used. Initial weights varied between 100-120 g (females) and 120-150 g (males). The age of the rats was about 6 weeks at the beginning of the experiments and 12 weeks at the end. The animals were of Specified Pathogen Free quality. Antibodies against the pneumonia virus of mice and the pin-worm *Syphacia muris* but against no other pathogens were found. The animals were kept in Macrolon type 3 cages on sterilized softwood granules as bedding. The animals were housed three per cage.

The animals were provided with RMH-TM pellets (Hope Farms b.v. Woerden, The Netherlands). The rats had free access to water with an increasing ethanol content reaching up to 15% w/v after 6 weeks. Details of the method of alcohol exposure are described in van de Wiel *et al.* (1990). Room temperature was regulated (22 ± 2°C); relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 7.00 a.m. and 7.00 p.m.. Series of 12, 20 or 24 rats were used. Each series was divided in four groups: male alcohol rats, male control rats, female alcohol rats, female control rats.

One group of rats was orally treated with B(*a*)P in the last week of ethanol treatment. They received 5 mg B(*a*)P per kg bodyweight daily (in olive oil), or olive oil only, during 7 days.

Preparation of microsomes

Six weeks after the start of the alcohol treatment, the rats were anaesthetized with pentobarbital and the livers were perfused in situ with ice-cold 0.9% NaCl solution. The livers were excised, weighed, collected in ice-cold 0.25 M sucrose, sliced and homogenized in 3 volumes ice-cold 0.25 M sucrose using a Potter-Elvehjem glass teflon homogenizer. The cell debris and nuclei were removed at a first centrifugal run at 600*g* in a Heraeus Christ minifuge (Gonzalez *et al.*, 1988) and mitochondria were removed by centrifugation for 20 min at 9000*g* in an IEC B20. The floating fat layer was removed and the underlying supernatant fraction decanted and homogenized. The post-mitochondrial fraction was centrifuged at 105000*g* for 75 min at 0-4°C in an IEC B50 ultracentrifuge. The pellet was resuspended in ice-cold 1.15% KCL, the volume adjusted to the original liver weight. The suspension was distributed in samples of 1 ml in small plastic vials, frozen in liquid nitrogen and stored at -80°C until use.

Enzyme measurements

Cytochrome P450 and protein content. Spectral analysis for cytochrome P450 content was performed according to the standard procedure (Rutten *et al.*, 1987). Protein concentrations of the samples were determined using the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V Boehringer) as the protein standard.

Aniline-4-hydroxylase (An-4-H) was determined with a modified method based on Ishidate *et al.* (1978). The reaction mixture contained 10 mM aniline, 0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, 1 unit glucose-6-phosphate dehydrogenase, 10 mM nicotinamide, 100 mM tris-acetatebuffer of pH 7.9 and 100 µl microsomes in a final volume of 1 ml. The reaction was carried out for 20 minutes at 37°C with moderate shaking and stopped by addition of 0.5 ml 20% ice-cold trichloroacetic acid. After centrifuging for 5 min at 800*g*, a 1 ml aliquot of the supernatant was taken out and 0.5 ml 10% Na_2CO_3 was added, followed by 1 ml of 2% phenol in 0.2 N NaOH. The resulting blue color was measured at 630 nm after 30 minutes.

Ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-deethylase (PROD) were measured as described by Burke et al. (1985).

Testosterone hydroxylation. Determinations in microsomal preparations were carried out in a 1 ml incubation mixture containing potassium phosphate buffer (50mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1mM), $NADP^+$ (1mM), glucose-6-phosphate (5mM), glucose-6-phosphate dehydrogenase (1 unit/ml), testosterone (250 µM) and 200-300 µg microsomal protein. Incubation mixture and microsomes were mixed in an ice-cooled container. Reactions were started by heating the mixture in a 37°C shaking water bath and stopped after 15 min by addition of 6 ml dichloromethane. Extraction and subsequent analysis of metabolites by HPLC were performed as described by Wortelboer *et al.* (1990). 11ß-OHT was used as internal standard.

Glutathione S-transferase (GST). Quantification of glutathione S-transferases was performed as described by Bogaards *et al.* (1989). In short, liver cytosol (1 ml) was applied on S-hexylglutathione affinity chromatography columns. The eluate, containing the GST-isoenzymes, was applied to wide pore-reversed phase HPLC (Vydac 201TP5 (200 X 3 mm d.d.), which was eluted with a gradient of water and acetonitrile both containing 0.1 % trifluoroacetic acid. The GST subunits, eluting as discrete peaks were quantified by peak area integration and comparison with known amounts of purified GST isoenzymes. Enzymatic activities were determined using 1-chloro-2,4-dinitrobenzene as substrate, according to Habig *et al.* (1974).

Epoxide hydrolase. The activity of epoxide hydrolase, performed by the addition of water to 1,2-epoxyethylbenzene (styrene oxide), was quantified by measuring the concentration of the produced phenylglycol (1-phenyl-1,2-ethandiol), according to Splinter *et al.* (1990).

Statistical analysis

Results are presented as mean values ± standard deviation. Statistical analysis was carried out using the Statistical Analysis System package on a VAX6410 minicomputer The procedure used was general linear model for analysis of variance with unequal cells A P-value <0.05 was considered to be of statistical significance With the analysis of variance procedure, it was possible to take four groups of rats into account (male ethanol, male control, female ethanol, female control)

RESULTS

The rats consuming drinking water containing up to 15% ethanol during six weeks showed a significant (P = 0.008) increase in microsomal P-450 content (nmol/mg protein) (Table 1) For male rats there was an increase of 38% and for females an increase of 14% due to the alcohol treatment. This increase enhanced the existing sex difference in P450 between males and females (P = 0 0001) Protein concentrations also showed a significant increase after ethanol treatment (P < 0 0001) No significant sex differences were observed (Table 1)

Rats	n	Protein (mg/ml) ± sd	P450 (nmol/mg protein) ± sd
M 0% EtOH	6	15 6 ± 1 2	0 77 ± 0 07
F 15% EtOH	6	18 0 ± 2 2*	1 06 ± 0 13*
M 0% EtOH	6	13 5 ± 3 0	0 65 ± 0 05 **
F 15% EtOH	6	21 3 ± 1 7*	0 74 ± 0 04* **

Table 1 Effect of ethanol treatment on hepatic protein and cytochrome P450 content in liver microsomes from male and female rats

* Value significantly different for ethanol treated and non-treated rats

**Value significantly different for male and female rats

Aniline hydroxylase activity was increased significantly in ethanol treated rats (Table 2). In male rats the increase was 57% and in female rats 38%. This enzyme activity also differed significantly (P = 0.001) between both sexes.

Rats	n	*p* -aminophenol formed (nmol/minxmg protein) ± sd
M 0% EtOH	5	0 65 ± 0 10
M 15% EtOH	5	1 02 ± 0 24*
F 0% EtOH	5	0 48 ± 0 03 **
F 15% EtOH	5	0 66 ± 0 12* **

Table 2 Effect of ethanol treatment on aniline-4-hydroxylase activity in liver microsomes from male and female rats * Value significantly different for ethanol treated and non-treated rats
**Value significantly different for male and female rats

Ethanol treatment affected neither EROD, nor PROD activity of male and female rats (Table 3) After treatment with B(*a*)P, with or without ethanol, EROD activity was enhanced by a factor six

Rats	n	EROD (nmol/minx mg protein)± sd	PROD (nmol/minx mg protein)± sd
M 0% EtOH	5	0 20 ± 0 08	0 069 ± 0 01
M 15% EtOH	5	0 25 ± 0 10	0 065 ± 0 02
M EtOH+B(a)P	3	1 42 ± 0 26***	
F 0% EtOH	4	0 21 ± 0 07	0 049 ± 0 01**
F 15% EtOH	4	0 20 ± 0 01	0 052 ± 0 01
F EtOH+B(a)P	3	1 15 ± 0 18***	

Table 3 Effect of ethanol treatment on ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-deethylase (PROD) activity in liver microsomes from male and female rats
** Value significantly different for male and female rats
***Values significantly different for B(*a*)P treated rats

In males, the formation of 6ß-hydroxytestosterone (6ß-OHT) was decreased by 25% after ethanol treatment In females, 12ß-OHT was significantly decreased to 50% of the initial value With all metabolites, except for 15ß-OHT and 19-OHT,

differences between the sexes were observed (Table 4) In males 6ß-OHT, 16α-OHT, 16ß-OHT, 2α-OHT and androstenedione were formed in larger quantities In females the formation of 19-OHT, 7α-OHT and 12ß-OHT was greater than in males

Rats (n=5)	M 0% EtOH	M 15% EtOH	F 0% EtOH	F 15% EtOH
testosteron metabolite (µmol/minx mg protein) ±sd				
15ß-OHT	0 02±0 00	0 02±0 01	0 02±0 00	0 03±0 01
6ß-OHT	0 28±0 06	0 21±0 04*	0 02±0 00**	0 02±0 01
19-OHT	0 81±0 11	0 65±0 22	1 12±0 23	0 82±0 49
7α-OHT	0 12±0 01	0 14±0 02	0 33±0 03**	0 36±0 08
16α-OHT	1 34±0 18	1 35±0 34	0 00 **	0 01±0 00
16ß-OHT	0 02±0 00	0 02±0 01	0 01±0 00**	0 01±0 00
12ß-OHT	0 00	0 00	2 72±1 00**	1 46±0 29*
2α-OHT	14 3±2 15	15 1±3 42	0 00 **	0 00
androstene-dione	0 60±0 07	0 61±0 11	0 08±0 00**	0 09±0 03

Table 4 Effect of ethanol treatment on testosterone hydroxylase activities (µmol/minxmg protein) in liver microsomes from male and female rats *Value significantly different for ethanol-treated and non-treated rats ** Value significantly different for male and female rats

Using CDNB as a substrate, we found the activity of glutathione S-transferase to be significantly higher in male than in female rats. No influence of ethanol treatment could be detected (Table 5). When the amounts of the different glutathione S-transferase subunits were measured, we generally observed a slight decrease in the ethanol-treated groups Subunit 1 was significantly decreased after ethanol treatment with 20% in males and 27% in females (P = 0 048) (Table 5) Although the total amount of GST per mg protein did not differ significantly between the sexes, several subunits did. Subunits 1,3 and 4 were found at higher levels in males, subunit 2 in females.

Rats (n=3)	M 0% EtOH	M 15% EtOH	F 0% EtOH	F 15% EtOH
CDNB (μmol/minx mg protein) ± sd	11.9±0 9	10 6±1.1	10.0±1 2**	9 5±0 5
subunit GST (μg subunit/ mg protein) ± sd				
1	10.8±2.9	8 5±1 6*	8.5±1 2**	6 2±0.7*
2	11 0±2 1	11.0±1 0	22 3±0 7**	19 3±2 3
3	5 3±1 3	4 9±0 0	2 2±0 2**	2 0±0.1
4	4 1±1 1	3 6±0 2	2.3±0 4**	2.1±0 1
6	0 2±0.1	0 2±0 1	0.3±0 1	0 2±0 1
total	31.5±7 3	28 1±1.9	35.5±1 3	29.9±3 0

Table 5 Effect of ethanol treatment on total glutathione S-transferase (GST) activity (CDNB as substrate) and subunit composition of glutathione transferase in liver cytosol from male and female rats *Value significantly different for ethanol treated and non-treated rats ** Value significantly different for male and female rats

Epoxide hydrolase was measured in rat liver cytosol and in rat liver microsomes (Table 6). Cytosolic epoxide hydrolase activity was not influenced by ethanol treatment Microsomal epoxide hydrolase was significantly decreased to 70% of its initial value after ethanol treatment ($P < 0.0001$) in male and female rats. In liver microsomes, there was also a significant difference between the sexes, males showing a 20% higher activity than females ($P < 0.001$).

Rats		n	phenylglycol formed (nmol/minx mg protein) ± sd	
			cytosolic	microsomal
M	0% EtOH	5	1 5±0 1	9 8±1 0
M	15% EtOH	5	1 3±0 1	6 8±0 9*
F	0% EtOH	5	1.4±0 2	7 4±0 9**
F	15% EtOH	5	1 4±0 2	5 3±0 7*

Table 6 Effect of ethanol treatment on epoxide hydrolase activity in liver microsomes and cytosol from male and female rats *Value significantly different for ethanol treated and non-treated rats ** Value significantly different for male and female rats

DISCUSSION

Biotransformation enzymes were measured in liver fractions from male and female rats to estimate whether the effects observed earlier of ethanol consumption on the pharmacokinetics and the biotransformation of B(*a*)P could be explained.

So far, most studies on the effects of ethanol treatment focused either on changes in liver morphology and function as in alcoholists (Lieber, 1984), or on the inducing capacity of ethanol on specific isoenzymes of P450 (Koop & Coon, 1986). We have measured the influence of ethanol treatment on a broad spectrum of enzymes that could be involved in or associated with the biotransformation of benzo(*a*)pyrene. In our ethanol treatment model the animals consume a moderate amount of ethanol. As we described earlier, this does not lead to considerable liver damage Also the growth of the rats is not significantly influenced (van de Wiel, 1990). Our treatment, comparable in the last three weeks to the intake in human 'social' drinking (Zentella de Pina *et al.*, 1989) leads to an increase of P450 content This increase can be largely attributed to a rising amount of P4502E1 (Koop & Coon, 1986), and is measured as a maximal two-fold increase in aniline-4-hydroxylase activity as was also described by Liu *et al.* (1975) and Prasad *et al.* (1985).

EROD activity was not changed after ethanol treatment This activity is associated with P4501A1, so there are no indications for a change in P4501A1 activity. However, when several doses of B(*a*)P were given, with or without ethanol, the inducing effect of B(*a*)P was clear and EROD activity increased with a factor six.

PROD (P4502B1/2B2) activity was not changed by ethanol treatment as was described for other specific P4502E1 inducers. After treatment of rats with acetone, Puccini et al. (1989) found no effect on PROD activity.

Testosterone metabolism is differentially influenced by ethanol treatment. A 25% decrease in the formation of 6ß-OHT was measured in males, representing the activity of the male specific P450's 3A2 and 2C13. In females, 12ß-OHT was lowered to 50% of the initial value. In non-induced rats, the constitutive sex-specific enzymes (Kato *et al.*, 1986; van de Wiel *et al.*, 1992) and not P4501A1 (Thomas *et al.*, 1981) are probably responsible for most of the B(*a*)P biotransformation activity. We also observed this in our *in vitro* study (van de Wiel *et al.*, 1992). Sex differences in testosterone metabolism were described by Kobliakov (1991). He found 16α-OHT and 6ß-OHT formation to be higher in males. We found five of the nine testosterone metabolites formed in larger amounts in males than in females. In females two metabolites were higher than in males.

The phase II enzymes which are important in B(*a*)P biotransformation, glutathione S-transferase and epoxide hydrolase, were both affected by ethanol treatment. Gonzalez (1988) measured a decrease of GST and GSH after acute ethanol treatment. Other authors reported that GST and GSH maximally increased after 2 weeks of ethanol treatment and did not differ from the controls from 6 weeks onward (Mierha Yang & Carlson, 1991; Muñoz *et al.*,1987). In our experiments the total activity of GST was decreased, but not significantly. The amount of subunit 1 of GST was significantly decreased in males and females, whereas the other remained unchanged. Up to now at least eight GST subunits have been identified and characterized. On the basis of their primary structure, they belong to 4 gene families, namely the alpha family (subunits 1,2 and 8), the mu family (subunits 3,4 and 6), the theta family (subunit 5) and the pi family (subunit 7). The pi family (GST 7-7) was described to be the most important enzyme in conjugating anti-BPDE (7ß,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene) and other electrophilic metabolites of benz(*a*)anthracene and chrysene (Jernström *et al.*, 1989). In that case a reduction in subunit 1 should have little impact on the amount of anti-BPDE conjugated. The relative proportion of subunit composition of GST between adult male and female rats found by us was the same as described by Igarachi (1987).

Epoxide hydrolase (EH) catalyzes the formation of diols from B(*a*)P-epoxides and the formation of tetrols from B(*a*)P-dihydrodiolepoxides. Epoxide hydrolase activity was decreased considerably by ethanol treatment. In *in vivo* and *in vitro* studies we earlier observed a decreased formation/excretion of B(*a*)P phenol and diol metabolites (9-hydroxy-B(*a*)P, 9-hydroxy-B(*a*)P, B(*a*)P-4,5-dihydrodiol, B(*a*)P-7,8-dihydrodiol, B(*a*)P-9,10-dihydrodiol (van de Wiel *et al.*, 1992; van de Wiel *et al.*, 1993). In a study by Selkirk (1975), inhibition of epoxide hydrolase was correlated with an increase of phenol

metabolites, while diols disappeared The decrease in EH activity measured in our present study therefore cannot be the source of a decreased formation of both phenols and diols. However, it is conceivable that this could have an effect on the amount of reactive *anti*-BPDE formed.

We practised a mild induction with a moderate amount of ethanol in the drinking water, comparable with 'social' drinking in humans. This caused moderate effects on the biotranformation enzymes. Increases and decreases in activity varied from 20-50%. However, these small effects on enzyme activity could have large consequences on bioactivation of compounds like B(*a*)P.

The differences we found earlier in excretion of B(*a*)P metabolites between ethanol and control rats can be linked to the enzyme measurements that are described in this paper. Bioactivation of B(*a*)P seems to be lowered after ethanol induction The meaning of this kind of observations for the human situation is not fully clear In our rat studies no evidence is obtained to support the possibility of enhanced bioactivation of B(*a*)P due to chronic alcohol use as a mechanism to enhance the risk of alcohol consuming people to develop cancer (IARC, 1988). There have been several suggestions about this (Lieber, 1984) Nevertheless no significant differences in AHH activity were measured in human liver samples of people that consumed alcohol or not (Rubin & Lieber, 1968) A change in phase II metabolism, for instance a decrease in epoxide hydrolase activity could be important.

In conclusion, it was shown in rats that long-term ethanol treatment induces 2E1, and does not alter 1A1 or 2B1/2B2 Reduction of 2C13 and 3A2 related activity is observed. This correlates with the lowered formation/excretion of certain B(*a*)P phenol and diol metabolites after long-term ethanol treatment observed *in vivo* and *in vitro* in our earlier work. A considerable reduction of epoxide hydrolase was also measured

REFERENCES

Bogaards, J J P , van Ommen, B , van Bladeren, P J An improved method for the separation and quantification of glutathione S-transferase subunits in rat tissue using high-performance liquid chromatography J Chromatography 474 435-440, 1989

Burke, M D , Thompson, S , Elcombe, C R., Halper, J , Haaparanta, T , Mayer, R.T Ethoxy-, pentoxy-, and benzyloxyphenoxazones and homologues A series of substrates to distinguish between different induced cytochromes P450 Biochem Pharmacol 34 3337-3345, 1985

Capel, I D , Jenner, M , Pinnock, M H , Williams, D C The effect of chronic alcohol intake upon the hepatic microsomal carcinogen-activation system Oncology 35 168-170,1978

Gonzalez, J , Muñoz, M E , Marin, M I , Collado, P S , Fermoso, J , Esteller, A Influence of acute ethanol administration on hepatic glutathione metabolism in the rat Alcohol 5 103-106, 1988

Habig, W H , Pabst, M J , Jakoby, W B Glutathione S-transferases The first enzymatic step in mercapturic acid formation J Biol Chem 249 7130-7139, 1974

IARC Alcohol drinking IARC Monographs on the evaluation of carcinogenic risks to humans vol 44, IARC, Lyon, France, 1988

Igarashi, T , Irokawa, N , Ono, S , Ohmori, S , Ueno, K , Kitagawa, H Difference in the effects of phenobarbital and 3-methylcholanthrene treatment on subunit composition of hepatic glutathione S-transferase in male and female rats Xenobiotica 17(2) 127-137, 1987

Ishidate, K , Yoshida, M , Nakazawa, Y Effect of typical inducers of microsomal drug-metabolizing enzymes on phospholipid metabolism in rat liver Biochem Pharmacol 27 2595-2603, 1978

Jernström, B , Dock, L , Hall, M , Mannervik, B , Tahir, M K , Grover, P L Glutathione transferase catalyzed conjugation of benzo(a)pyrene 7,8-diol 9,10-epoxide with glutathione in human skin Chem -Biol Interactions 70 173-180, 1989

Johansson, I , Ekstrom, G , Scholte, B , Puzycki, D , Jornvall, H , Ingelman-Sundberg, M Ethanol-, fasting- and aceton-inducible cytochromes P-450 in rat liver regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies Biochemistry 27 1925-1934, 1988

Kato, R , Yamazoe, Y , Shimada, M , Murayama, N , Kamataki, T Effect of growth hormone and ectopic transplantation of pituitary gland on sex-specific forms of cytochrome P450 and testosterone and drug oxidations in rat liver J Biochem 100 895-902, 1986

Kobliakov, V , Popova, N , Rossi, L Regulation of the expression of the sex-specific isoforms of cytochrome P-450 in rat liver Eur J Biochem 195 585-591, 1991

Koop, D R , Coon, M J Ethanol oxidation and toxicity role of alcohol P-450 oxygenase Alcoholism Clin Exp Res 10s, 1986

Lieber, C S , DeCarli, L M Ethanol oxidation by hepatic microsomes, adaptive increase after ethanol feeding Science 162 917-918, 1968

Lieber, C S Metabolism and metabolic effects of alcohol Med Clin N Am 68,3-31 1984

Lieber, C S Interaction of ethanol with drugs, hepatotoxic agents, carcinogens and vitamins Alcohol Alcoholism 25(2/3) 157-176, 1990

Liu, S J , Ramsey, R K , Fallon, H J Effects of ethanol on hepatic microsomal drug-metabolizing enzymes in rat Biochem Pharmacol 24 369-378, 1975

Lowry, O H , Rosebrough, N J , Farr, A L , Randall, R J Protein measurement with the Folin phenol reagent J Biol Chem 193 265-275, 1951

Mierha Yang, C , Carlson, G P Effects of ethanol on glutathione conjugation in rat liver and lung Biochem Pharmacol 41(6/7) 923-929, 1991

Muñoz, M E , Martin, M I , Fermoso, J , Gonzalez, J , Esteller, A Effect of chronic ethanol feeding on glutathione and glutathione-related enzyme activities in rat liver Drug Alc Dep 20 221-226, 1987

Onishi, K ,Lieber,C S Reconstitution of the microsomal ethanol-oxidizing system J Biol Chem 252 7124-7131, 1977

Prasad, J S , Crankshaw, D L , Erichson, R.R., Elliott, C E , Husby, A D , Holtzman, J L Studies on the effect of chronic consumption of moderate amounts of ethanol on male rat hepatic microsomal drug metabolizing activity Biochem Pharmacol 34(19) 3427-3431, 1985

Puccini, P , Fiorio, R., Longo, V , Gervasi, P G High affinity diethylnitrosamine-deethylase in liver microsomes from acetone-induced rats Carcinogenesis 10(9) 1629-1634, 1989

Rubin, E , Lieber, C S Hepatic microsomal enzymes in man and rat Induction and inhibition by ethanol Science 162 691, 1968

Rutten, A J J L , Falke, H E , Catsburg, J F , Topp, R., Blaauboer, B J , van Holsteijn, I , Doorn, L , van Leeuwen, F X R. Interlaboratory comparison of total cytochrome P450 and protein determinations in rat liver microsomes Arch Toxicol 61 27-33, 1987

Ryan, D E , Ramanathan, L , Iida, S , Thomas, P E , Haniu, M , Shively, J E , Lieber, C S , Levin, W Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid J Biol Chem 260 6385-6393, 1985

Seitz, H K , Garro, A J , Lieber, C S Effect of chronic ethanol ingestion on intestinal metabolism and mutagenicity of benzo(a)pyrene Biochem Biophys Res Comm 85 1061-1066, 1978

Selkirk, J K , Croy, R G , Gelboin, H V Isolation by high pressure liquid chromatography and characterization of benzo(a)pyrene-4,5-epoxide as a metabolite of benzo(a)pyrene Arch Biochem Biophys 168 322-326, 1975

Splinter, K , Bauer, D , Seidel, W Epoxide hydrolase, its function and determination of its activity in rat liver Exp Pathol 39 195-196, 1990

Todorovic, R , Devanesan P D , Cavalieri, E L , Rogan, E G , Park, S S , Gelboin, H V A monoclonal antibody to rat liver cytochrome P4502C11 strongly and regiospecifically inhibits constitutive benzo(a)pyrene metabolism and DNA binding Mol Carcinogen 4 308-314, 1991

Thomas, P E , Reik, L M , Ryan, D E , Levin, W Regulation of three forms of cytochrome P450 and epoxide hydrolase in rat liver microsomes J Biol Chem 256(2) 1044-1052, 1981

Tuyns, A J Alcohol and cancer Proc Nut Soc 49 145-151, 1990

van de Wiel, J A G , Duijf, C M P , Pertijs, J C L M , Copius Peereboom-Stegeman, J H J , Bos, R P Growth and liver morphology after long-term ethanol consumption of rats Lab Anim 24 265-272, 1990

van de Wiel, J A G , Fijneman, C M P , Anzion, R B M , Theuws, J L G , Bos, R P Excretion of benzo(a)pyrene and metabolites in urine and feces of rats, Influence of route of administration, sex and long-term ethanol treatment Toxicology, in press, 1993

van de Wiel, J A G , Meuwissen, M , Kooy, H , Fijneman, P H S , Noordhoek, J , Bos, R P The influence of long-term ethanol treatment on in vitro biotransformation of benzo(a)pyrene in microsomes of liver, lung and small intestine from male and female rats Biochem Pharmacol 44(10) 1977-1984, 1992

Wortelboer, H M , de Kruif, C A , van Iersel, A A J , Falke, H E , Noordhoek, J , Blaauboer, B J The isoenzyme pattern of cytochrome P450 in rat hepatocytes in primary culture comparing different enzyme activities in microsomal incubations and in intact monolayer Biochem Pharmacol 40 2525-2534, 1990

Wynder, E L , Bross, I J , Feldman, R L A study of etiological factors in cancer of the mouth Cancer 10 1300-1323, 1957

Wynder, E L , Bross, I J A study of etiological factors in cancer of the esophagus Cancer 14 389-414, 1961

Zentella de Pina, M , Vellalobos-Molina, R , Saavedra-Molina, A , Riveros-Rosas, H , Pina, E Effects of moderate chronic ethanol consumption on rat liver mitochondrial functions Alcohol 6 3-7, 1989

CHAPTER VI

INFLUENCE OF LONG-TERM ETHANOL TREATMENT ON DNA-ADDUCT FORMATION IN LIVER AND INTESTINAL EPITHELIAL CELLS OF RATS AFTER ONE ORAL DOSE OF BENZO(*A*)PYRENE

J.A.G. van de Wiel[1],N. Ajubi[1],J.W.M. Gielen[1],
M.-J.S.T. Steenwinkel[2], R.A. Baan[2], R.P. Bos[1]

1. Department of Toxicology, Faculty of Medical Sciences,
University of Nijmegen, P.O. Box 9101,
6500 HB Nijmegen
The Netherlands

2. Department of Genetic Toxicology
TNO Medical Biological Laboratory
Rijswijk,
The Netherlands

Submitted

ABSTRACT

The influence of long-term ethanol exposure of rats on the formation of benzo(*a*)pyrene [B(*a*)P]-DNA adducts in rat liver and intestinal epithelial cells has been studied. One group of rats had free access to tap water without ethanol, the other group received tap water with an ethanol content increasing from 0 to 15% over 3 weeks, and kept at 15% for another 3 weeks. Rats of both groups were treated with a single dose of B(*a*)P by gavage (50 mg/kg bodyweight). Liver and intestinal samples were analyzed at 6, 12, 18, 24 and 36 h after B(*a*)P administration by use of the ^{32}P-postlabelling method. DNA adducts were detected at each of these time points. In liver and intestine, 5 and 4 different adducts were detected, respectively. Adducts B, C and F were detected both in liver and in intestinal cells. In liver, adducts C and F were quantitatively the most prominent. In intestine, adduct B was the predominant adduct. The level of DNA adducts was significantly higher in intestinal epithelial cells than in liver cells. The effect of ethanol on adduct levels in liver was opposite to that in intestinal cells. In liver of long-term ethanol-treated rats, a clear maximum of the levels of adducts C and F was reached at 24 h after the administration of B(*a*)P. In non-treated rats, a plateau was reached after 12 h at a much lower level. In intestinal cells of ethanol-treated and non-treated rats, the levels of adducts B and C reached a maximum at about 18 h. However, the adduct levels in ethanol-treated rats were decreased at 24 h, whereas those in non-treated rats were still high at this time point. Thirty-six hours after administration of B(*a*)P, the adduct levels in both groups of rats were at a low level again. The enhancing effect of ethanol treatment on the level of adduct C in liver was significant. This paper demonstrates that long-term ethanol consumption has an effect on B(*a*)P-DNA adduct formation in rats.

INTRODUCTION

The impact of life style factors on toxicokinetics of foreign compounds is clearly recognized. The main, (non-professional) life-style associated effects stem from the use of alcohol and tobacco, the intake of medication and general dietary habits (Founier, 1986). Effects of alcohol use on the toxicokinetics of polycylic aromatic hydrocarbons (PAHs) are of interest because PAHs are ubiquitous environmental contaminants and components of cigarette smoke. The risks of cancer of the larynx, pharynx and oesophagus have been found to be additive or multiplicative for people who smoke as well as drink alcohol (IARC, 1988).

Ethanol is known to give rise to a broad spectrum of effects (Lieber, 1984). In animal experiments it has not shown to be a carcinogen, but ethanol may alter the extent and/or the route of absorption, biotransformation and excretion of environmental carcinogens, such as N-nitrosamines (Swann *et al.*, 1987) and

PAHs (Lieber, 1984). PAHs have to be activated by the monooxygenase system to become genotoxic (Brookes and Lawley, 1964). Ethanol acts as an inducer of the monooxygenase-linked cytochrome P450 complex but the specific ethanol-inducible P450 isozyme (P4502E1) does not have a high affinity for PAHs (Rubin and Lieber, 1968, Onishi and Lieber, 1977, Koop and Coon, 1986, Johansson *et al.*, 1988). Conceivably, the additive and/or multiplicative effect of drinking and smoking on cancer risks could be attributed to a changed metabolism of PAHs by the ethanol-induced P450-monooxygenases. Indeed, a higher activity of aryl hydrocarbon hydroxylase in microsomes of female rat liver and gut and of male rat liver was observed by some authors, after giving ethanol in a synthetic liquid diet (Seitz *et al.*, 1978, 1981, 1981a). An enhanced metabolism of benzo(*a*)pyrene [B(*a*)P] in the cheek-pouch epithelium, but not in the liver of Syrian golden hamsters was measured after long-term ethanol treatment (Murphy & Hecht, 1986). Other authors, however, described a decrease or no change in the in vitro metabolism of B(*a*)P by rat and hamster liver microsomes isolated from ethanol-pretreated animals (DeMarco & McCoy, 1984). We also measured a decreased formation of B(*a*)P metabolites *in vivo* and in liver microsomes after long-term ethanol pretreatment (van de Wiel *et al.*, 1992, 1993).

It is known that DNA adducts may play an important role in the initiation of B(a)P-induced cancer (Swenberg *et al.*, 1985). Because we were interested in the effect of chronic ethanol consumption on B(*a*)P-DNA adduct formation, we examined the levels of DNA adducts in liver and small intestine of ethanol-pretreated and non-pretreated rats at various time intervals after a single oral dose of B(*a*)P.

MATERIALS AND METHODS

Chemicals

Ethanol absolute GR, supplied by Merck (Darmstadt, Germany) was used for the pretreatment. Benzo(*a*)pyrene (CAS 50-32-8), micrococcal endonuclease, spleen exonuclease and trypsin inhibitor were purchased from Sigma (St. Louis, MO, USA). Heparin was from Organon Teknika (Boxmeer, The Netherlands). Nuclease P_1, RNAse A, RNAse T_1 and proteinase K were purchased from Boehringer (Mannheim, Germany). T4 polynucleotide kinase was from Biolabs (Amersfoort, The Netherlands). PEI-cellulose thin-layer chromatography (TLC) sheets were from Merck (Darmstadt, Germany). (^{32}P) ATP (specific activity >5000 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, Ireland). XAR-5 film was from Kodak (Driebergen, The Netherlands).

Animals and treatment

Homebred Cpb:WU (Wistar) male rats were used. Their initial weights varied between 120-150 g. The age of the rats was about 6 weeks at the beginning of the experiments and 12 weeks at the end. The animals were of SPF quality (antibodies against PVM virus and the pin-worm *Syphacia muris* were found, no other pathogens). The animals initially were kept in Macrolon type 3 cages on sterilized softwood granules as bedding, 3 animals per cage. During the last week of the ethanol treatment the animals were housed individually in stainless steel metabolism cages.

The animals were provided with RMH-TM pellets (Hope Farms b.v. Woerden, The Netherlands). A group of 12 rats had free access to tap water without ethanol, another group of 12 animals received tap water with an ethanol content increasing from 0 to 15% over 3 weeks, and then kept at 15% for another 3 weeks. The body weights at the beginning of the ethanol pretreatment did not differ significantly in both groups. After the pretreatment, the average body weight of the rats in the ethanol group was 9.6% less than that of the control group (not statistically significant). The relative liver weights (liver weight/body weight) of the rats of the two groups showed no difference. Details of the method of alcohol exposure have been described previously (van de Wiel *et al.*, 1990). Room temperature was regulated (22 ± 2°C), relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 7.00 a.m. and 7.00 p.m.

After 6 weeks of ethanol pretreatment, 10 rats of each group received one oral dose of B(*a*)P, administered by gavage (50 mg/kg in 1 ml olive oil), the other 2 received olive oil only. At 6, 12, 18, 24 and 36 h after B(*a*)P treatment, 2 animals of each group were sacrificed. Two (one ethanol-treated, one non-treated) of the animals that received the vehiculum only, were sacrificed at 6 h, the other two at 24 h after treatment. During this period, the ethanol treatment was continued.

The rats were anaesthetized with pentobarbital and perfused with ice-cold 0.9% NaCl via the heartchamber until the liver and lungs were decoloured. Livers were excised, frozen in liquid nitrogen and stored at -80°C until use. An intestinal segment consisting of the first 20 cm distal to the pylorus was immediately excised, perfused free of intestinal content with ice-cold 15% KCl solution containing 0.05 M Tris-HCl buffer (pH 7.4), slit open and washed. The upper villous layer of the mucosa was removed by scraping with the edge of a glass slide. The epithelial cells were suspended in 4 ml of the KCl-Tris buffer. To this suspension, trypsin inhibitor (5 mg/g wet weight of small intestine), glycerol (20% v/v, final concentration) and heparin (3 U/ml) were added, as described by Stohs *et al.* (1976). The cells were frozen in liquid nitrogen and stored at -80C° until use.

DNA isolation

Livers were thawed in 250 mM sucrose/ 100 mM EDTA, pH 7 4 (9 ml/g liver) and thoroughly minced in a Potter Elvejhem apparatus (Braun, Melsungen) while kept in ice The nuclear fraction was isolated by centrifugation for 1 min at 4000 *g* The pellet was resuspended in sucrose/EDTA (9 ml/gram tissue) and centrifuged for 10 min at 700 *g* The pellet was then resuspended in 250 mM sucrose/25 mM EDTA/ 1% Triton X-100 (5ml/gram tissue) and incubated for 30 min at 4°C Chromatin was isolated by centrifugation (10 min at 700 *g*), resuspended in ice-cold 10mM Tris-HCl/25mM EDTA pH 7 4 (TE-buffer, 5 ml per gram tissue) and again centrifuged at 700 *g* and 4°C The last two steps were performed thrice Finally, the pellet was resuspended in TEN-buffer pH 7 4 (20 mM Tris-HCl, 1 mM EDTA, 0 1 M NaCl)(2 5 ml/gram tissue) This was also the first step (after thawing) in the isolation of DNA from cells of the small intestine To this suspension an equal volume of TEN-buffer with 1% SDS and proteinase K (100 µg/ml) was added, followed by overnight incubation at 37°C

To purify the DNA, extractions were performed with phenol, phenol chloroform isoamyl alcohol (25 24 1) and chloroform isoamyl alcohol (24 1) The procedure for the three extractions was identical To the water-phase containing the DNA, an equal volume of organic solute was added This mixture was shaken for 10 min at room temperature and then centrifuged for 10 min at 700 *g* at 4°C The upper layer was removed and extracted again Finally, ice-cold ethanol was added to the aqueous phase to precipitate the DNA The DNA was collected around the end of a heat-sealed glass pipette It was washed twice with 70% ethanol and dried in a vacuum desiccator The DNA was resuspended overnight in TE-buffer pH 7 4 (5ml per gram tissue)

After addition of 7 5 µl RNAse A (10000 U/ml) and 7 5 µl RNAse T_1 (10 µg/ml) the DNA suspension was incubated for 3 h at 37°C Per ml suspension 0 1 ml 10x-concentrated TEN-buffer, 0 1 ml 10% SDS and 10 µl proteinase K (10 mg/ml) were added This was incubated at 37°C for 1 h The DNA was isolated by extractions, precipitation, washing and drying as above The DNA was resuspended overnight in Millipore-filtered (MQ) water

The DNA concentration in the various samples was determined spectrophotometrically (1 mg/ml of DNA= 20 absorbance units at 260 nm)

^{32}P-postlabelling

This assay for adducts in DNA was performed according to the nuclease P1-modified procedure described by Reddy and Randerath (1986) and Randerath *et al* (1989) Isolated DNA was digested with micrococcal nuclease and spleen phosphodiesterase From each sample an aliquot was taken for quantification of the DNA (see below) The rest was dephosphorylated with nuclease P1 The nucleotides derived from 8-12 samples of DNA (5 µg DNA/sample) were labelled simultaneously (3 25 MBq [^{32}P] ATP/sample) by incubation with T4

polynucleotide kinase for 30 min at 37°C and -per sample- applied to 10cm * 20cm polyethylenimine (PEI)-cellulose TLC sheets A paper wick was attached to the top of each sheet Chromatography occurred for 60 h in 1 1 M LiCl (D1) at room temperature The paper wick was removed and discarded After two washes with water the chromatograms were developed in 8 5 M urea, 3 M lithium formate, pH 3 5 (D3) for 3 5 h and then in 8 5 M urea, 0 8 M LiCl, 0 5 M Tris-HCl, pH 8 0 (D4) for 3 h In order to remove any remaining impurities, the chromatograms were developed for 16 h in 0 35 M $MgCl_2$ (D5, in the direction of D1) The adducts were localized by autoradiography by use of a Kodak XAR-5 film and an intensifying screen, at -70^{0}C The adduct areas on the PEI-cellulose sheets detected by autoradiography were cut out and the radioactivity was determined by liquid scintillation counting The level of DNA adducts was calculated on the basis of the analysis of standard samples containing known amounts of modifications (Steenwinkel *et al*, in preparation)

Aliquots of the digests obtained after micrococcal and spleen nuclease treatments (see above), were analysed for DNA content by fast-protein-liquid-chromatography (FPLC) on a Mono Q anion-exchange column (Pharmacia) For each postlabelling sample, the input DNA was quantified on the basis of the area of the 3'-GMP-peak in the FPLC elution pattern (19 9% of total nucleotides is guanine) For quantification of this peak a standard calibration curve was used

Statistical analysis

Results are presented as mean values Statistical analysis was carried out by use of the SAS (Statistical Analysis System) package on a VAX6410 minicomputer The procedure used was GLM (General Linear Model) for analysis of variance with unequal cells A p-value < 0 05 was considered to indicate statistical significance

RESULTS

Rats that had been chronically treated with ethanol for 6 weeks via the drinking water, were compared with untreated rats The *in vivo* binding of B(*a*)P metabolites to DNA of rat liver and intestinal epithelium was examined after administration of a single oral dose of B(*a*)P After DNA isolation, the ^{32}P-postlabelling assay was applied to detect and quantify DNA adducts Autoradiograms obtained upon ^{32}P-postlabelling of DNA from liver and intestinal cells are shown in Figures 1 and 2, respectively

In liver, the total adduct level was maximal 0 3-0 6 fmol/µg DNA Five adduct spots were detected, coded B,C,F,Y and Z (Figure 1)

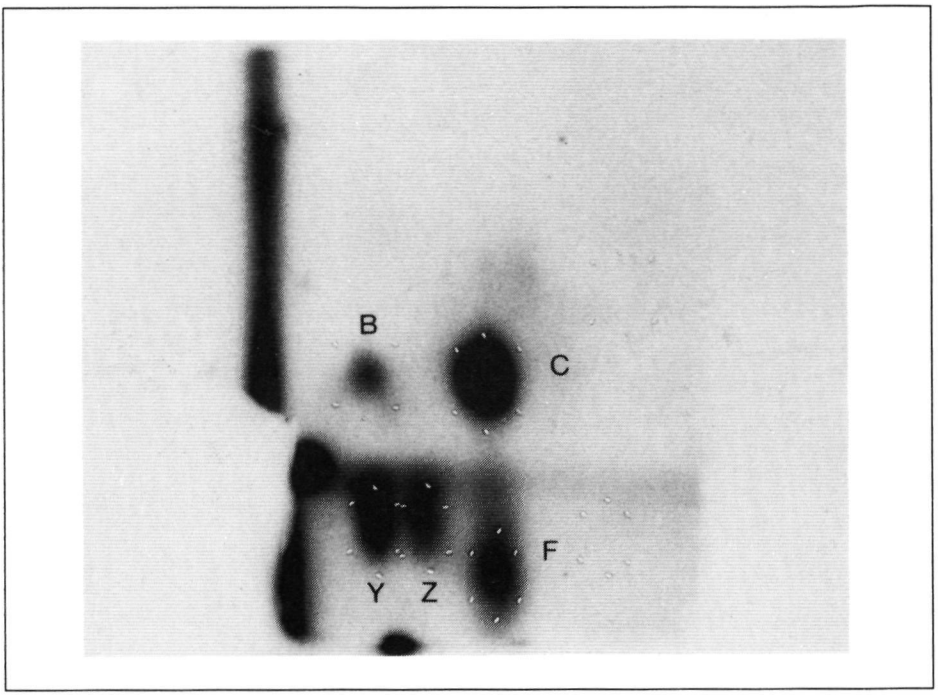


Figure 1. Autoradiogram of B(*a*)P-modified rat liver DNA, obtained from a rat treated orally with B(*a*)P at 100 mg/kg body weight. The positions of spots F,Y and Z indicate that migration of these components has occurred in the direction of D1.

The levels of adducts B,C and F at various time points after B(*a*)P treatment are shown in Figure 3. Adducts C and F were quantitatively the most prominent ones. In the ethanol-treated rats, a maximum of the levels of adducts C and F was reached at 24 h after the administration of B(*a*)P. In non-treated rats, in general lower levels than those in the ethanol-treated rats were observed, with a kind of plateau reached after 12 h. For adduct C, when all time points were considered, ethanol-treated rats had a significantly higher level ($p=0.02$). At 6 and 24 h the p-values were 0.03 and 0.003 respectively. Adduct F was significantly higher in ethanol-treated rats at 6 h ($p=0.04$). At 36 h after B(*a*)P administration the level of all three adducts still seemed to be increased in ethanol-pretreated rats, although the differences are not statistically significant.

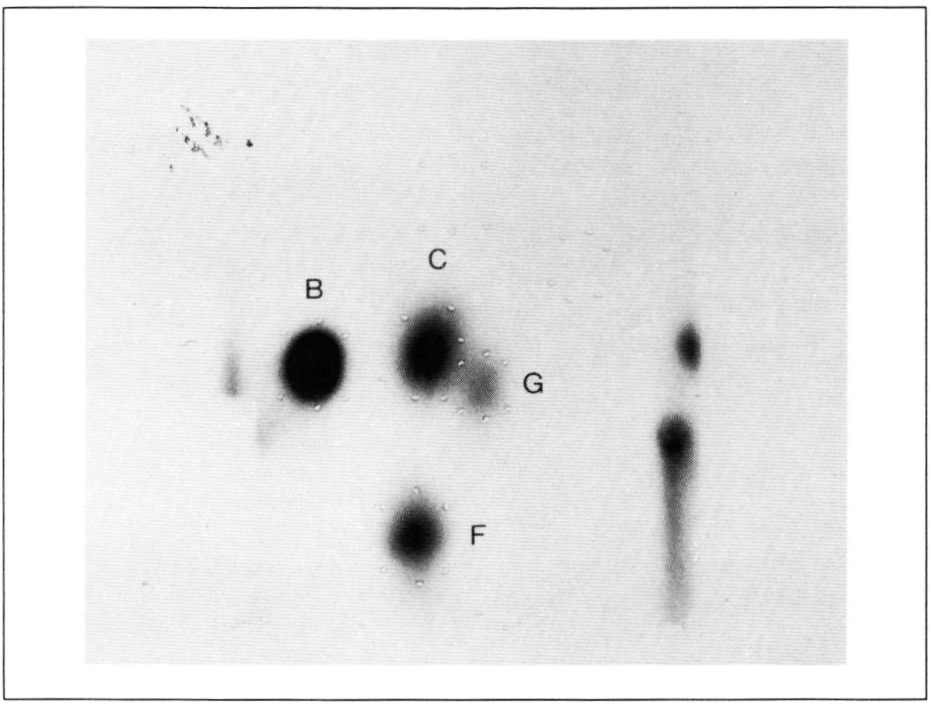


Figure 2. Autoradiogram of ^{32}P-postlabeled DNA. Sample of B(*a*)P-modified intestinal epithelial cell DNA, obtained from a rat treated orally with 100 mg/kg body weight

In the epithelial cells of the rat intestine the adduct levels were much higher than those in liver DNA. At 18 h, the total adduct level was 3.5 fmol/μg DNA. Four adducts were detected, coded B,C,F and G (Figure 2). In intestinal DNA, adduct B was dominant. The levels of adducts B, C and F at various time points after B(*a*)P treatment are shown in Figure 4.

At first no differences between ethanol-treated and control rats were observed. In both cases the levels of adducts B and C strongly increased, in a comparable way, between 0 and 18 h after B(*a*)P administration. Between 18 and 24 h, however, the adduct levels in ethanol-treated rats decreased, while those in non-treated rats stayed high. Thirty-six hours after administration of B(*a*)P, the adduct levels were low again in both groups of rats. In intestinal DNA, adduct F was the minor adduct; there were no differences between the two groups.

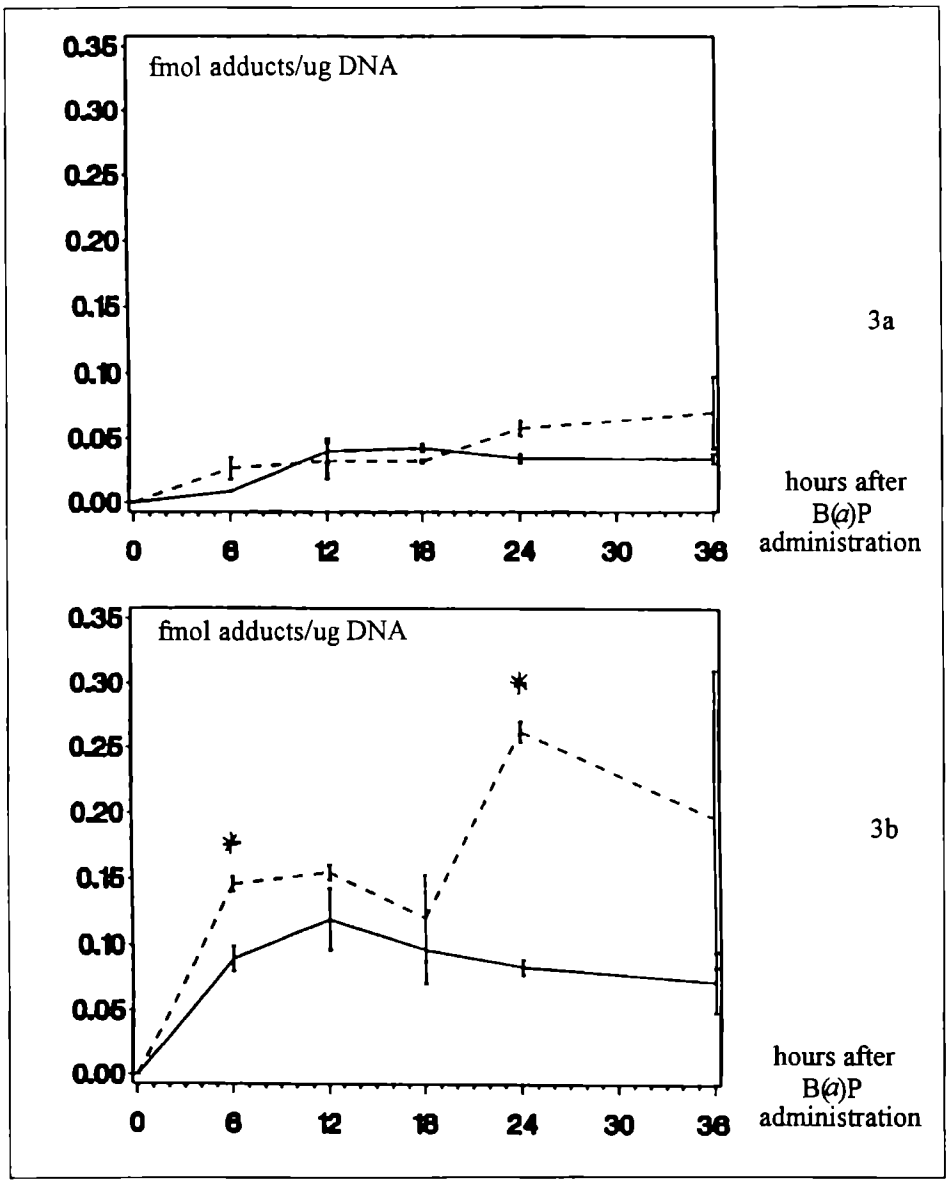


Figure 3 Levels of B(*a*)P adducts in rat liver after administration of 50 mg B(*a*)P/kg body weight. Each point is the mean of two rats ± standard error of the mean. * Means a significant difference between ethanol-treated and non-treated rats (a) Adduct B, (b) adduct C, (c) adduct F. (—) control rats, (----) ethanol-treated rats

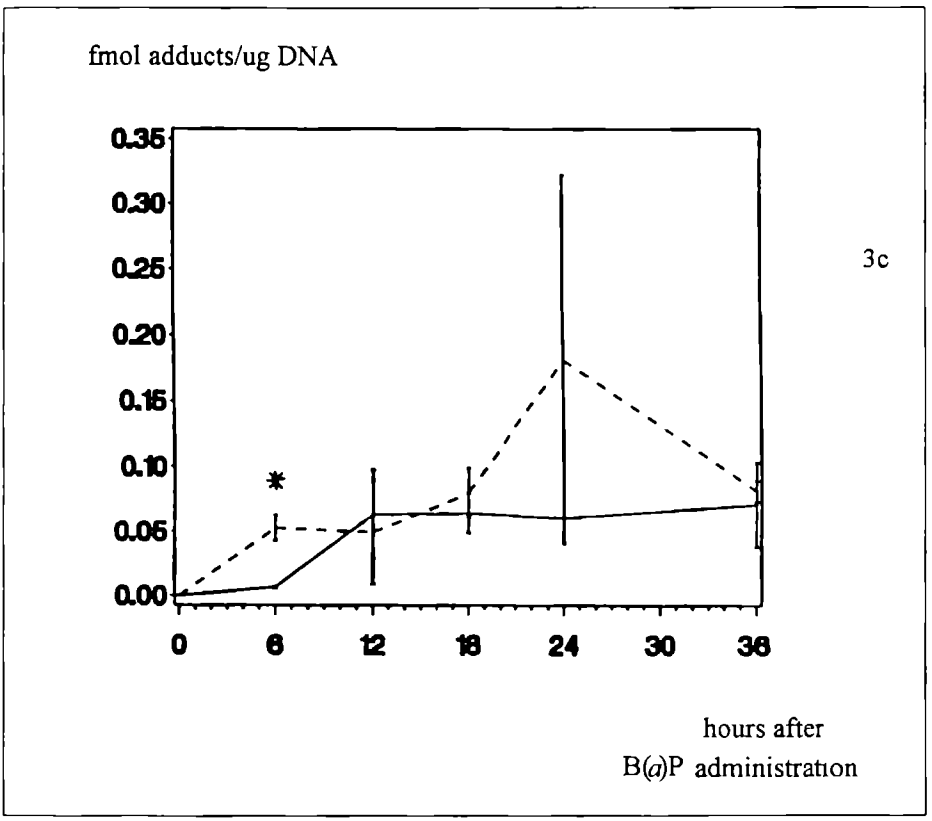


Figure 3 Levels of B(*a*)P adducts in rat liver after administration of 50 mg B(*a*)P/kg body weight Each point is the mean of two rats ± standard error of the mean * means a significant difference between ethanol-treated and non-treated rats (a) Adduct B, (b) adduct C, (c) adduct F (——) control rats, (-----) ethanol-treated rats

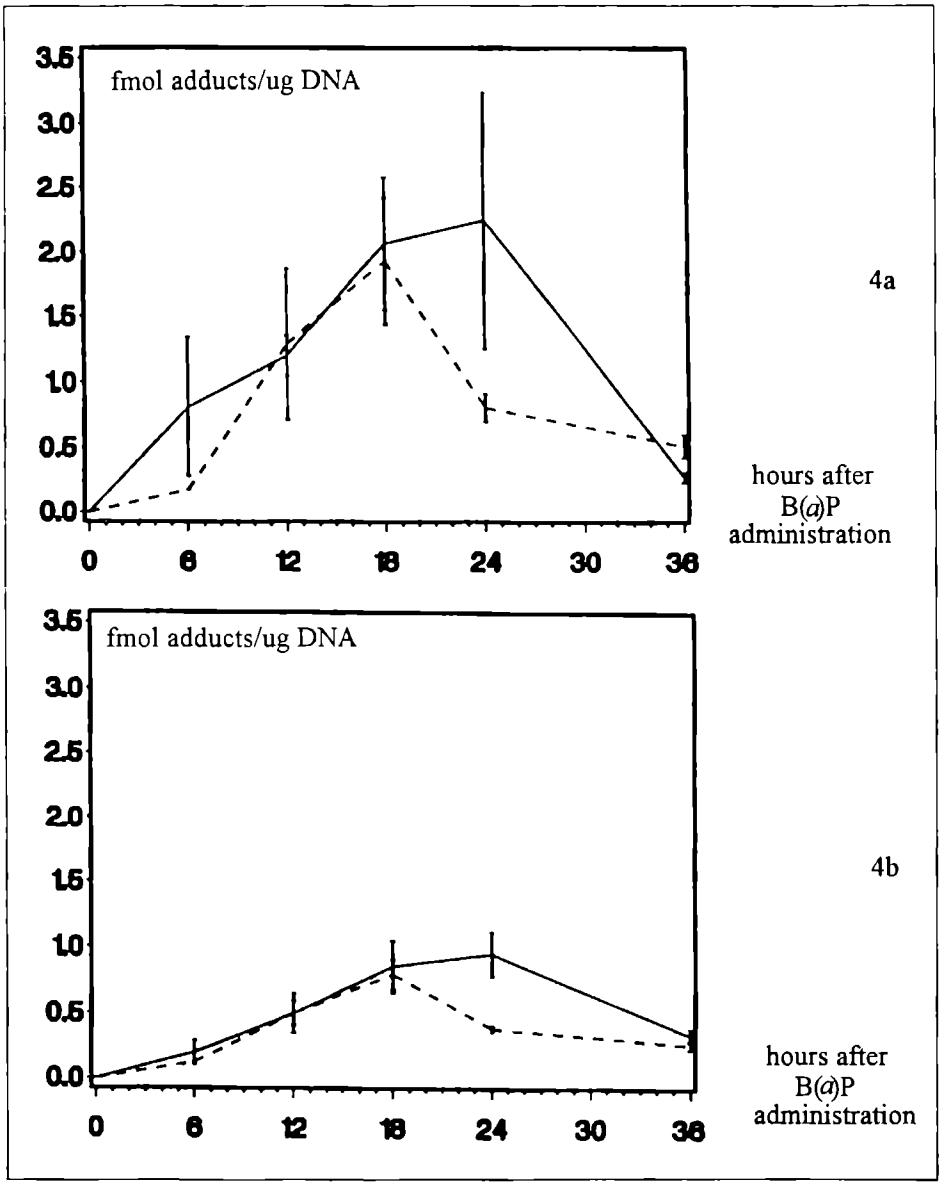


Figure 4. Levels of B(*a*)P adducts in rat intestine after administration of 50 mg B(*a*)P/kg body weight. Each point is the mean of two rats ± standard error of the mean * Means significant difference between ethanol-treated and non-treated animals (a) Adduct B, (b) adduct C, (c) adduct F. (——) control rats, (-----) ethanol-treated rats

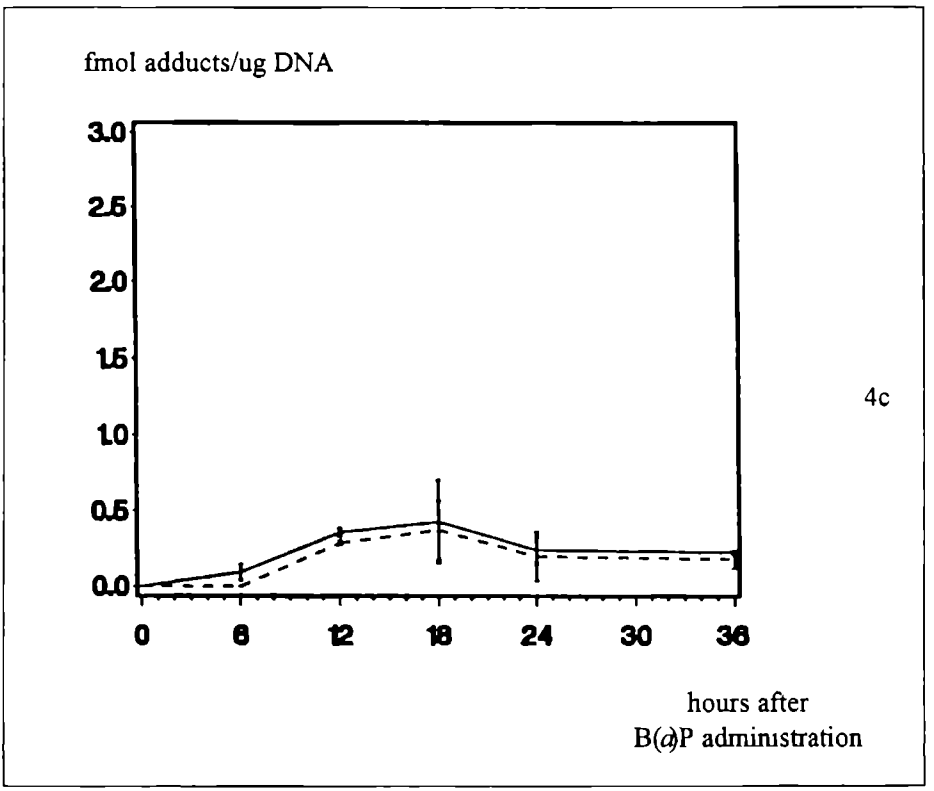


Figure 4 Levels of B(*a*)P adducts in rat intestine after administration of 50 mg B(*a*)P/kg body weight Each point is the mean of two rats ± standard error of the mean * Means a significant difference between ethanol-treated and non-treated rats (a) Adduct B, (b) adduct C, (c) adduct F (——) control rats, (-----) ethanol-treated rats

DISCUSSION

Both in rat liver and intestinal epithelium, B(*a*)P-DNA adducts were already detected at 6 h after the administration of B(*a*)P. The adduct levels in gut were generally about 10-fold higher than those in liver. This difference can probably be explained by the fact that after oral administration B(*a*)P reaches the intestinal epithelium as the first metabolizing tissue. Autoinduction can reinforce higher adduct levels in gut (Aito, 1974).

Both in liver and intestine the quantitatively predominant adduct C could be tentatively identified as the B(*a*)P desoxyguanosine-N^2-adduct (B(*a*)P-N^2-dG).

Adduct C cochromatographed with the adduct derived from the reaction of B(*a*)P-7,8-dihydrodiol-9,10-epoxide with calf thymus DNA *in vitro*.

The identity of the other adducts is not known; they could be formed upon binding of the B(*a*)P-7,8-dihydrodiol-9,10-epoxide to other sites in the DNA, for instance adenosine. It is also possible that they represent adducts of other B(*a*)P metabolites like the (+)- and (-)-enantiomers of the *anti*-dihydrodiol epoxide of B(*a*)P (Canella *et al.*, 1991).

In comparable studies (Ross *et al.*, 1990, Willems *et al.*, 1991,) the value of total adduct levels is of the same order of magnitude as reported here.

Melikian *et al.* (1990) compared ethanol-treated hamsters with non-treated animals. A significant increase in the DNA-adduct level was seen in the cheek pouch.

In our study, ethanol treatment affected the level of certain DNA adducts in liver and intestine. A significant enhancement was found for the main adduct in liver after ethanol treatment. In the gut ethanol treatment tended to have the opposite effect. Possibly, ethanol treatment affects gut epithelial tissue in such a way that it becomes more permeable for non-metabolized B(*a*)P (Persson, 1991). This would give rise to a lower adduct level in the intestinal epithelium, but at the same time the amount of B(*a*)P that reaches the liver may be increased and consequently more reactive metabolites could be formed there.

In our earlier studies (van de Wiel *et al.*, 1992) a lowered formation of phenolic B(*a*)P metabolites in liver microsomes was found, this correlates with an enhanced DNA-adduct formation. In small intestine we measured an increase of the formation of phenolic metabolites and a decreased DNA-adduct level. Ethanol treatment influences enzymes like epoxide hydrolase that deactivate reactive intermediates of B(*a*)P. In the liver this enzyme is lowered after ethanol treatment (van de Wiel *et al.*, in press). It is conceivable that this effect enhanced the B(*a*)P-DNA adduct formation in the liver.

Adduct levels are a result of formation and breakdown. Breakdown of adducts is caused by DNA repair mechanisms, necrosis of cells and cell turnover. Epithelial cells of the gastro-intestinal tract have a relatively high turnover. The faster decline in adduct levels in the small intestine of ethanol-treated rats could be partly explained by the fact that this turnover is even further enforced by ethanol. DNA adducts can be easily diluted in this way. In contrast DNA adducts are better preserved in the liver with its low cell turnover.

It is yet to be assessed what our observations mean when transferred to the human situation. Our rat studies did not provide evidence to support the hypothesis that alcohol drinkers' increased risk of cancer is due to enhanced bioactivation of B(*a*)P (IARC, 1988). However, a change in phase II metabolism might play a role. The decrease in epoxide hydrolase activity we measured earlier can be related to the increased amounts of DNA adducts in the livers of ethanol-treated rats reported here.

REFERENCES

Aito, A 1974 Different elimination and effect on mixed function oxidase of 20-methyl cholanthrene after intragastric and intraperitoneal administration Res Com Chem Path Pharm 9 701-710

Brookes,P and Lawley,P D 1964 Nature 202 781-784

Canella,K , Peltonen,K and Dipple,A 1991 Identification of (+) and (-) anti benzo[a]pyrene dihydrodiol epoxide-nucleic acid adducts by the 32P-postlabelling assay Carcinogenesis 12 1109-1114

DeMarco,G J and McCoy,G D 1984 Comparison of effect of chronic ethanol consumption by Syrian golden hamsters on liver metabolism and mutagenicity of benzo(a)pyrene and N-nitrosopyrrolidine Proc Am Assoc Cancer Res 25, 320

Fournier,P E and Thomas,G 1986 The impact of life style factors and consumption of pharmaceutical drugs on toxicokinetics In 'Health surveillance of individual workers exposed to chemical agents' (W R F Notten, R F M Herber, W J Hunter, A C Monster, R.L Zielhuis eds) pp 22-31 Springer-Verlag, Berlin

IARC 1988 Alcohol drinking IARC Monographs on the evaluation of carcinogenic risks to humans Alcohol Drinking vol 44, IARC, Lyon, France

Johansson,I , Ekstrom,G , Scholte,B , Puzycki,D , Jornvall,H and Ingelman-Sundberg,M 1988 Ethanol-, fasting- and aceton-inducible cytochromes P-450 in rat liver regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies Biochemistry 27 1925-1934

Koop,D R and Coon,M J 1986 Ethanol oxidation and toxicity role of alcohol P-450 oxygenase Alcoholism Clin Exp Res 10 suppl

Lieber,C S 1984 Metabolism and metabolic effects of alcohol Med Clin N Am 68 3-31

Melikian,A A , Fudem Goldin,B , Prahalad,A K and Hecht,S S 1990 Modulation of benzo[a]pyrene-DNA adducts in hamster cheek pouch by chronic ethanol consumption Chem Res Toxicol 3 139-143

Murphy,S E and Hecht,S S 1986 Effects of chronic ethanol consumption on benzo(a)pyrene metabolism and glutathion-S-transferase activities in Syrian Golden Hamster cheek pouch and liver Cancer Res 46 141-146

Onishi,K and Lieber,C S 1977 Reconstitution of the microsomal ethanol-oxidizing system J biol chem 252 7124-7131

Persson,J 1991 Review Alcohol and the small intestine Scand J Gastroenterol 26 3-15

Randerath,K , Randerath,E , Danna,T F , van Golen, K L and Putman,K L 1989 A new sensitive 32P-postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5'-monophosphates Carcinogenesis 10 1231-1239

Reddy,M V and Randerath,K 1986 Nuclease P1-mediated enhancement of sensitivity of 32P-postlabeling test for structurally diverse DNA adducts Carcinogenesis 7 1543-1551

Ross,J , Nelson,G , Kligerman,A , Erexson,G , Bryant,M , Earley,K , Gupta,R. and Nesnow,S 1990 Formation and persistence of novel benzo(a)pyrene adducts in rat lung, liver, and peripheral blood lymphocyte DNA Cancer Res 50 5088-5094

Rubin,E and Lieber,C S 1968 Hepatic microsomal enzymes in man and rat Induction and inhibition by ethanol Science 162 691

Sato,A , Yonekura,I , Asakawa,M , Nakahara,H , Nakajima,T , Ohta,S , Shirai,T and Ito,N 1986 Augmentation of ethanol-induced enhancement of dimethylnitrosamine and diethylnitrosamine metabolism by lowered carbohydrate intake Jpn J Cancer Res (Gann) 77 125-130

Seitz,H K , Garro,A J and Lieber,C S 1978 Effect of chronic ethanol ingestion on intestinal metabolism and mutagenicity of benzo(a)pyrene Biochem Biophys Res Comm 85 1061-1066

Seitz,H K , Garro,A J and Lieber, C S 1981a Sex dependent effect of chronic ethanol consumption in rats on hepatic microsome mediated mutagenicity of benzo(a)pyrene Cancer Lett 13 97-102

Seitz,H K , Garro,A J and Lieber,C S 1981b Enhanced pulmonary and intestinal activation of procarcinogens and mutagens after chronic ethanol consumption in the rat Europ Jour Clin Inves 11 33-38

Stohs,S J , Grafström,R C , Burke,M D , Moldeus,P W and Orrenius,S G 1976 The isolation of rat intestinal microsomes with stable cytochrome P450 and their metabolism of benzo(a)pyrene Arch Biochem Biophys 177 105-116

Swann,P F , Graves,R J and Mace,R 1987 Effect of ethanol on nitrosamine metabolism and distribution Implications for the role of nitrosamines in human cancer and for the influence of alcohol consumption on cancer incidence Mutat Res 186 261-267

Swenberg,J A , Richardson,F C , Boucheron,J A and Dyroff, M C 1985 Relationships between DNA adduct formation and carconogenesis Environm Health Persp 62 177-183

van de Wiel,J A G , Duijf,C M P , Pertijs,J C L M , Copius Peereboom- Stegeman,J H J and Bos, R P 1990 Growth and liver morphology after long-term ethanol consumption of rats Lab Anim 24 265-272

van de Wiel,J A G , Meuwissen,M , Kooy,H , Fijneman,P H S , Noordhoek,J and Bos,R P 1992 Influence of long-term ethanol treatment on in vitro biotransformation of benzo(a)pyrene in microsomes of the liver, lung and small intestine from male and female rats Biochem Pharmacol 44 1977-1984

van de Wiel,J A G , Fijneman,P H S , Duijf,C M P , Anzion,R B M , Theuws,J L G and Bos,R P 1993 Excretion of benzo(a)pyrenen and metabolites in urine and feces of rats Influence of route of administration, sex and long-term ethanol treatment Toxicology 80 103-115

van de Wiel, J A G , Fijneman,P H S , Teeuw,K B , van Ommen,B , Noordhoek,J , Bos,R P 1993 Influence of long-term ethanol treatment on rat liver biotransformation enzymes Alcohol (in press)

Willems,M I , Roggeband,R , Baan,R A , Wilmer,J W G M , de Raat,W K and Lohman,P H M 1991 Monitoring the exposure of rats to benzo[a]pyrene by the determination of mutagenic activity in excreta, chromosome aberrations and sister chromatid exchanges in peripheral blood cells, and DNA adducts in peripheral blood lymphocytes and liver Mutagenesis 6 151-158

CHAPTER VII

EFFECT OF LONG-TERM ETHANOL ADMINISTRATION COMBINED WITH SHORT-TERM B(*A*)P TREATMENT ON DNA-ADDUCT LEVELS IN SEVERAL ORGANS OF RATS

J.A.G. van de Wiel[1], Y. Jansma[1], J.J.A. Muller[2], E.D. Kroese[2], R.P. Bos[1]

1. Department of Toxicology, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen The Netherlands

2. Laboratory of Carcinogenesis and Mutagenesis, Institute of Public Health and Environmental Hygiene P.O. Box 1, 3720 BA Bilthoven The Netherlands

Submitted

ABSTRACT

The effect of a combination of short-term oral benzo(*a*)pyrene [B(*a*)P] treatment (13 times 5 mg/kg) and prolonged oral ethanol administration (15% in the drinking water) on B(*a*)P-DNA adducts was investigated with the ^{32}P-postlabeling assay. The four most prominent B(*a*)P-DNA adducts were measured in liver, small intestine, forestomach and oesophagus. The adduct level was highest in liver > small intestine > forestomach > oesophagus. In liver, adduct 4 was most prominent, while in forestomach, small intestine and oesophagus adduct 2 was most important. Adduct 2 co-chromatographed with the benzo(*a*)pyrene-7,8-diol-9,10 epoxide (BPDE)-DNA adduct. In the liver, one adduct was significantly ($P<0.05$) increased in ethanol- and B(*a*)P-treated rats compared to rats that only got B(*a*)P. In the small intestine one adduct and in the forestomach two adducts were significantly decreased. It is concluded that ethanol treatment has an organ specific effect on B(*a*)P DNA-adduct formation.

INTRODUCTION

Apart from other pathological consequences, alcohol-abuse is recognized as a major risk factor for cancer of the upper alimentary tract and the upper respiratory tract. Cancer of the large intestine, breast, pancreas and liver is also correlated with alcohol consumption (Fraumeni, 1979; Garro & Lieber, 1990; Lieber *et al.*, 1979).

Cigarette smoking is a well known risk factor for developing cancer too (IARC, 1986). Combined use of alcoholic beverages and tobacco has a synergistic effect on the risk of developing cancer in pharynx, larynx, oesophagus and oral cavity (Garro & Liber, 1990; Lieber *et al.*, 1979). About 76% of these cancers could be avoided if alcohol and tobacco use were given up (Rothman & Keller, 1972). The mechanism underlying this synergism has not been discovered yet, although various explanations have been suggested (Fraumeni, 1979; Garro & Lieber, 1979). In one of these hypotheses ethanol is supposed to act as a cocarcinogen by inducing metabolic enzymes. Ethanol is an inducer of several enzymes, including the mono-oxygenase system. This enzyme complex is known to be important in the biotransformation of several carcinogens (IARC, 1988; Ioannides & Parke, 1990). Some of these compounds are known to be present in tobacco, for example PAH (Polycyclic Aromatic Hydrocarbons) or nitrosamines (IARC, 1986). PAH are formed as products of the incomplete combustion of organic materials (Phillips, 1983). Apart from smoking, humans can be exposed to PAH orally through contamination of food or drinking water or, mostly in occupational situations, by inhalation and by skin contact (Kriek *et al.*, 1989). One of the 11 PAH known to be carcinogenic in experimental animals is

benzo(*a*)pyrene (B(*a*)P) (IARC, 1983). The ultimate carcinogenic metabolite of B(*a*)P is (±)7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene (BPDE). Following in vivo treatment, the major B(*a*)P metabolite DNA-adduct formed is (+)-7ß,8α dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro benzo(*a*)pyrene [(+)-anti-BPDE] bound by a trans addition to the exocyclic amino group of deoxyguanosine (N^2) (Bodell *et al.*, 1989). The formed adduct (BPDE-dG) can be found in tissues of exposed animals and cells of humans (Hall & Grover, 1990). Covalent binding to DNA (DNA-adducts) is a well known genetic endpoint for analyzing carcinogenic risks (Phillips, 1990). In a previous study (van de Wiel *et al.*, Influence of long-term ethanol pretreatment on DNA-adduct formation in liver and intestinal epithelial cells of rats after one oral dose of benzo(*a*)pyrene, *submitted*) the effect of prolonged ethanol administration on B(*a*)P induced DNA-adducts was studied in liver and small intestine of rats with the sensitive ^{32}P-postlabeling method. At several points in time, DNA-adducts were measured. 24 Hours after B(*a*)P administration the maximal B(*a*)P-DNA adduct level was achieved in all organs. The results showed that ethanol treatment of the rats resulted in an increase in adduct formation in the liver and a decrease in the small intestine. However, in that study, B(*a*)P was applied only once and it is known that B(*a*)P itself also induces the mono-oxygenase system. To make a better comparison with the "real-life situation", the present study was performed using short-term exposure with several daily doses, instead of a single B(*a*)P dose. All samples were taken 24 hours after the last B(*a*)P administration. DNA adducts were analyzed in oesophagus and forestomach as target organs for B(*a*)P/ethanol carcinogenicity and in liver and small intestine as the major metabolizing organs.

MATERIALS AND METHODS

Chemicals

1,2 Cyclohexamine diamine tetra-acetic acid (CDTA), natriumacetate, chloroform, ethanol, isopropanol, succinate, CaCl2 dihydrate, MgCl2 hexahydrate and bicine were purchased from Merck, Darmstadt, Germany. ^{32}P-Pi was from Amersham Corporation, Arlington Heights, Ireland. T4 polykinase was from Pharmacia, Uppsala, Sweden. Micrococcal endonuclease, Benzo(*a*)pyrene (CAS 50-32-8), Na-pyruvate (type II), Na-ADP (grade 1), dithiothreitol, ZnCl2, Spermine and Na-2'-deocyadenosine-3-monofosfate were obtained from Sigma Chemicals Co., St. Louis, USA. Proteinkinase K., (2x) Lysis buffer, chloroform and 70% phenol/chloroform/water were from Applied Biosystems, Foster City, USA. Other chemicals (or enzymes) were obtained from Boehringer-Mannheim, Germany.

Animals

16 Male homebred Cpb:WU (Wistar) rats (120-150 grams) were used. The age of the rats was about 6 weeks at the beginning of the experiment. The animals were

of Specified Pathogen Free quality. The only antibodies found, were those against the pneumonia virus of mice and the pin-worm *Syphacia muris*. During six weeks the animals were housed three per cage in Macrolon cages on sterilized softwood granules as bedding. During the last 3 weeks they were housed individually in stainless steel metabolism cages. Room temperature was regulated (22±2°C), relative humidity varied between 40 and 60%. The animals were exposed to artificial light between 7.00 a.m. and 7.00 p.m.. Food (RMH-TM pellets, Hope Farms BV, Woerden, the Netherlands) was given ad libitum. 8 rats had free access to tap water without ethanol. The other group of 8 rats received a rising percentage (w/v) of ethanol in their drinking (tap)water during three weeks: 4 days 2%, 3 days 4%, 4 days 6%, 3 days 8%, 4 days 10%, 3 days 12%. After that they received 15% ethanol in their drinking water during the rest of the experiment (van de Wiel *et al.*, 1990). Every other day drinking fluid was renewed. Benzo(*a*)pyrene, dissolved in olive oil, was administered orally in a dose of 5 mg/kg to all rats; 5 times in the 7th week, 5 times in the 8th week and 3 times in the 9th week. Ethanol treatment was continued during this period to the appropriate group.

The initial body weights of the animals in both groups did not differ significantly. After the treatment, the average body weight of the rats in the ethanol-treated group was significantly less ($P<0.01$) than the non-treated group although the intake of calories of both groups was comparable. The relative liver weights (liver weight/ body weight) of the two groups showed no significant difference.

24 Hours after the last B(*a*)P treatment all rats were anaesthetized with pentobarbital and perfused with ice-cold 0.9% NaCl via the left ventricle of the heart. Liver, small intestine, oesophagus and forestomach were isolated, frozen in liquid nitrogen and stored at -80°C.

Isolation of DNA

All organs were homogenized (15%) in Hanks solution. 100 µl α-Amylase (10.000 U/ml and 63 µl of the chelating agent CDTA (500 mM, pH=8.0) were pipetted into 1.5 ml of the homogenate and incubated for one hour at 37°C. Then 100 µl RNAse (RNAse T1 + RNAse A; both 1000 U/ml 30 mM sodium acetate buffer pH=6) was added to the homogenate. The samples were loaded onto the Nucleic Acid Extractor Model 340A (Applied Biosystems). The homogenate was enzymaticly digested in Lysis buffer (at 60°C) by RNAse and Proteinase K (1500 U dissolved in 12 ml sterile 20 mM TRIS-HCL pH=8.5) and extracted by 70% Phenol/chloroform/water (twice) and chloroform (once) at ambient temperature. DNA was precipitated in 300 mM sodium acetate (pH=5.5) by isopropanol, collected onto a filter and washed with ethanol. Finally, the DNA was dissolved in TRIS.HCL (5mM, pH 7.4).

The DNA concentration of the samples was determined by measuring UV absorbance (260 nm). Contamination of the sample with protein (280 nm) and

organic solvents (230 nm) was also checked. The average concentration of DNA (mg/ml TRIS-HCl) in liver samples was 0.63 mg/ml (sd = 0.11), in forestomach samples 0.31 mg/ml (sd = 0.14), in small intestine samples 0.9 mg/ml (sd = 0.4) and in oesophagus samples 0.22 mg/ml (sd = 0.12). Because the DNA concentration in the postlabeling assay should be between 0.250 mg/ml and 1.0 mg/ml, the oesophagus samples were concentrated to 0.385 mg DNA/ml by vacuum-drying.

Postlabeling assay

This was largely derived from Gupta and Randerath (1982,1985) with some modifications (SOP, 1991). With one labeling-assay 8 organs (4 B(*a*)P-treated rats and 4 B(*a*)P- and ethanol-treated rats) were analyzed. In every postlabeling, a standard (lung of rat treated with B(*a*)P) and a (TRIS-HCl) blank were also examined.

With 8 µl hydrolyse-mix (1.17 µl (6 µg/µl) micrococcal endonuclease 2.63 µl (2.67 µg/µl) spleen phosphodiesterase in 1.17 µl (360 mM) succinate pH=6.0, 1.17 µl (180 mM) $CaCl_2$ and 0.86 µl *aqua dest*), 5 µg DNA in 13 µl 5mM TRIS-HCl was hydrolysed to deoxyribonucleotide 3'-monophosphates (dNp) in 4 hours at 37°C.

To 15 µl of the 21 µl DNA-digest 1.2 µl Nuclease P1 (5 µg/µl) in 1.8 µl 0.3 mM $ZnCl_2$ with 0.417 M sodium acetate pH=5.0 was added. The samples were incubated at 37°C. After 40 minutes the reaction was stopped with 2.4 µl 0.5 M TRIS (free base).

To label the nucleotides, 20.4 µl DNA digest and 6 µl Kinasemix were mixed and incubated for 30 minutes at 37°C. The kinasemix contained: 2.4 µl kinase-buffer (0.1 M Bicine NaOH buffer, 0.1 M $MgCl_2$, 0.1 M Dithiothreitol and 10 mM Spermidine), 0.82 µl T4 Polynucleotidekinase (4.33 U), 75 uCi ^{32}P[ATP] and was completed with *aqua dest.* 22.4 µl of the labeled DNA was slowly applied to the origin of a TLC-sheet (Polychram cel 300 PEI Macherey-Nagel).

The original sheet (20 x 20 cm) was previously wetted in aqua dest, dried and cut along the line (0,16) - (20,16). On top of the now 16 x 20 cm sheet a "wick" (Whatman paper, 17 x 14 cm) was stapled on the short side. With a pencil the OR was stated at (14,14). The part of the sheet under the OR was soaked in D1 solvent (2.3 M NaH_2PO_4 pH=5.75), before the ^{32}P labeled nucleotides were added. The sheets were placed in a TLC-tank (Sigma). After a development in D1 for 23-24 hours, the sheets were cut from (0,16) to (16,16). Next the sheets were washed in a plastic tray with aqua dest (250 ml) on a shaker for 15 minutes (after 10 minutes aqua dest was renewed). After this the sheets (16 x 16 cm) were dried with a cold hair dryer and a wick of 17 x 10 cm was stapled on the opposite site. For a better separation of the adducts, the bottom of the sheets were soaked (1-2 cm) in aqua dest before placing the sheets in D2 (8.5 M urea, 3.6 M

lithium-formiate). After developing in the opposite direction of D1 for 14-15 hours (overnight), the wicks were removed and the same washing-procedure took place Without a wick, the sheets were placed in D3 (0 72 M Na_2HPO_4 H_2O, 0 45 M TRIS and 7.6 M Urea, pH=8 2) The direction of running was perpendicular to D1 and D2 When the solution reached the top of the sheets (mostly after 6-7 hours), the sheets were washed and dried again A wick of 17 x 10 cm was attached (OR right and down) and without wetting the bottom, the sheets were placed in D4 (1 7 M NaH_2PO_4 pH=6 0) for overnight development in the same direction as D3 Eventually (after D4) the wicks were cut off and the sheets were dried The OR's of the sheets were also removed because of the high amount of radioactivity The other edges of the sheets were marked with colored ^{14}C, the number of the sheet (1 to 10) was also indicated with ^{14}C The sheets were wrapped in foil and put into cassettes with radioactivity sensible layers After at least 24 hours the sheets were removed and the cassettes were screened on the Phosphorimager (Molecular Dynamics) The activity on each screen was measured and all data were stored on a 120 mbyte tape (3M)

Determination of nucleotides and Specific Activity

4 µl of hydrolysed DNA was diluted in 9254 µl TRIS (5 mM pH=7 4) This solution was mixed twice carefully and 5 µl was applied to an eppendorf cup In 2 other eppendorf cups 5 µl of a known amount of deoxyadenosine (which functions as standard for the amount of nucleotides) was pipetted The dAp solution was made by dissolving 6 6 mg 3'-dAp (Na+-salt) in 1 liter 5 mM TRIS-HCl pH=7 0, dAp was estimated spectrophotometrically using E_{max}=152000/M/cm at 260 nm This solution was diluted to 10 pmol in 25 µl aqua dest (1·50) Then 2.5 µl 10 mM bicine was applied to the nucleotide and dAp cups Together with the labeled DNA-samples, the nucleotide and dAp samples were labeled with 2 5 µl Kinase(^{32}P)mix and incubated After applying 22 4 µl ^{32}P-DNA to the PEI-sheets, the remaining 4 µl was used for checking the excess of ^{32}P[ATP] in the samples 22 Cups (2 for specific activity, 10 for nucleotides and 10 for examination of excess) were diluted 10000 times by adding first 990 µl 0.20 mM bicine-NaOH pH=9 6 and diluting 100 µl of this in 900 µl Bicine. 4 µl of this diluted solution was spotted on a TLC-sheet (Merck DC-plastikfolien PEI-cellulose F; 20 x 20 cm) The sheet was placed in a tank with 100 ml 0.12 M phosphate-buffer pH=6.1 for 1 5 hour After this time the sheet was taken out, dried, wrapped in plastic foil and stored till the adduct-sheets were finished. The sheet was screened using the Phosphorimager

Counting

Data were analyzed in ImageQuant (software going with the Phosphorimager) The level of radioactivity was measured in the blank-sample. This value was used as background-activity for every sheet The color range was set from

blank-activity to 50 times blank-activity. Each adduct on the 9 other sheets was encircled on the computer screen with the help of a mouse. The volume activity was determined by integration of each pixel of the circle. ATP,G,A,C,T and Pi were encircled too and the resulting 132 volumes (22 x 6) were integrated (without background subtraction). The mean recovery efficiency for the adducts was 54%.

Statistical evaluation

The four most important adducts were analyzed. The average of the adducts for ethanol-treated and control rats were compared and evaluated with the SAS (Statistical Analytical System) package on the VAX 6410. The procedure used was GLM (General Linear Model). A P-value <0.05 was considered to indicate statistical significance.

RESULTS

In the liver, small intestine and forestomach, 4 adducts could be determined. In the oesophagus only three of these adducts were found.

In both ethanol/B(*a*)P treated rats and in B(*a*)P-treated rats the same adduct spots were observed. The results are shown in Table 1.

	Liver		Forestomach		Small Intestine		Oesophagus	
Ethanol	-	+	-	+	-	+	-	+
adduct 1	31 (20)	8 (15)	55 (44)	24 (19)	40 (20)	21 (17)	3 (2)	5 (2)
adduct 2	76 (49)	79 (39)	166 (95)	67* (60)	232 (155	109 (75)	34 (15)	36 (24)
adduct 3	7 (6)	34* (30)	21 (12)	7* (8)	20 (11)	9* (6)	1 (2)	2 (4)
adduct 4	379 (203)	456 (173	45 (26)	20 (18)	53 (35)	56 (56)	-[b]	-

Table 1 RAL (Relative Adduct Level; adducts per 10^{10} nucleotides) in liver, forestomach, small intestine and oesophagus in ethanol/B(*a*)P and B(*a*)P treated rats[a]. Values are means of 5-8 samples for each adduct in each subgroup (standard deviation) *)Significantly different ($P<0.05$) from control rats (-) a) For ethanol and B(*a*)P treatment see Materials and Methods section b) No adduct measured

The total adduct level was highest in liver > small intestine > forestomach > oesophagus.

In liver, adduct 4 was the most prominent, while in the forestomach, small intestine and oesophagus adduct 2 was most important. Adduct 2 cochromatographed with the BPdG adduct. Adduct 3 was significantly ($P<0.05$) increased in ethanol/B(*a*)P-treated rats compared to B(*a*)P-treated rats.

In the small intestine adduct 3, and in the forestomach adducts 2 and 3 were significantly decreased in ethanol/B(*a*)P-treated rats compared to B(*a*)P-treated rats. The other adduct levels in forestomach and small intestine were also decreased, but not significantly.

In the oesophagus, ethanol treatment seemed to have no effect on B(*a*)P DNA-adduct formation.

The variation in adduct-values between individual rats was considerable for all organs. As can be seen in Table 2, the adduct percentage (percentage of one adduct compared to the total adduct level observed) was less variable. Ethanol treatment seemed to have no influence on the kind of adducts formed, but it did influence the amount of adducts formed. This effect was organ dependent.

	Liver		Forestomach		Small Intestine		Oesophagus	
Ethanol	-[a]	+	-	+	-	+	-	+
adduct 1	5 (4)	1 (2)	18 (6)	22 (4)	12 (4)	12 (6)	6 (6)	17 (21)
adduct 2	16 (4)	13 (2)	58 (4)	56 (5)	66 (7)	59 (10)	91 (10)	79 (21)
adduct 3	2 (2)	5 (3)	7 (1)	5 (3)	6 (2)	5 (1)	3 (4)	4 (9)
adduct 4	77 (8)	81 (5)	16 (5)	16 (5)	15 (5)	24 (12)	-[b]	-

Table 2: RAL as percentage of total (1-4) adducts. Values are expressed as mean (and standard deviation). a) For ethanol and B(*a*)P treatment see Materials and Methods section. b) No adduct measured

DISCUSSION

This study was performed to get more insight in the mechanism of interaction of ethanol and B(*a*)P. In a previous study (van de Wiel *et al.*, Influence of long-term ethanol treatment on DNA-adduct formation in liver and intestinal epithelial cells of rats after one oral dose of benzo(*a*)pyrene, *submitted*), using the same strain of rats and the same ethanol treatment procedure, it was found that after a single dose of B(*a*)P (50 mg/kg) individual and total adduct levels in liver were significantly increased. In small intestine, adduct levels were significantly decreased after ethanol pretreatment. Because B(*a*)P itself may induce biotransformational enzymes (Phillips, 1983) and multiple exposure is more realistic than a single exposure, the present study was performed subjecting rats to a subchronic B(*a*)P treatment (13 * 5 mg/kg).

In the present study, it was demonstrated that the adduct level was highest in liver > small intestine > forestomach > oesophagus. Ethanol pretreatment resulted in higher adduct levels in liver, while in forestomach and small intestine lower adduct levels were present. In the oesophagus no difference was detected. This is in agreement with the findings of the previous study. Ethanol treatment influenced the absolute adduct level, but not the kind of adducts formed. There are several ways of interaction between ethanol and B(*a*)P to enhance carcinogenesis as outlined by Villa *et al.* (1991) with respect to hepatocarcinogenisis. Alcohol can inhibit DNA repair and influence sister chromatid exchanges, exerting a promoting activity. There is also sound experimental evidence that alcohol can act as a promotor at metabolic level by inducing the biotransformation enzymes that turn a procarcinogen into a carcinogen. However, this is true for compounds like nitrosamines and small organocyclic molecules that are substrate for the ethanol inducible P4502E1. B(*a*)P is no substrate for this isozym (Ryan *et al.*, 1985), so the enhanced formation of B(*a*)P-DNA adducts in liver cannot be explained by this proces. On the contrary, in our earlier studies (van de Wiel *et al. 1992),* a lowered formation of phenolic B(*a*)P metabolites in liver microsomes of ethanol-treated rats was found. This correlates with an enhanced DNA-adduct formation. In small intestine we measured an increase of the formation of phenolic metabolites and a decreased DNA-adduct level after ethanol treatment.

Alcohol can also influence the enzymes that deactivate a reactive intermediate, like epoxide hydrolase. In the liver, this enzyme is lowered after ethanol treatment (van de Wiel *et al.*, 1993). It is conceivable that this enhanced the B(*a*)P DNA adduct levels in the liver as was measured.

Ethanol treatment seems to have an organ dependent effect on B(*a*)P DNA-adduct formation. Adduct levels are a result of formation and breakdown. Breakdown of adducts is caused by DNA repair mechanisms, necrosis of cells and cell turnover. The difference between effects in the liver and that in the

forestomach and small intestine could be explained by the fact that epithelial cells of the gastro-intestinal tract have a relatively high turnover. DNA-adducts can be easily diluted and this can be enforced by ethanol. In contrast, the liver has a low cell turnover, so DNA adducts are probably conserved better. Opposite effects in different organs were also reported by Brauze *et al.* (1991). They used genetically responsive [to aryl hydrocarbon hydroxylase induction, C57BL/10 (B10)] and nonresponsive [DBA/2 (D2)] mice. The responsive mice had lower levels of adducts in the liver but higher ones in the small intestine than the non-responsive mice.

Large differences between the highest and the lowest value of one adduct in one organ of rats of the same group were found. Part of this variation is due to the postlabeling assay. The inter-labeling variance can range to 40%. Biological variance also contributes to this variability.

It is concluded that ethanol treatment has an organ specific effect on B(*a*)P adduct levels. Ethanol treatment results in a decrease in individual adduct levels in forestomach and small intestine and an increase in liver adduct levels No significant differences in adduct levels in oesophagus were detected. The kind of adduct does not seem to alter after ethanol treatment, pointing to a quantitative, rather than a qualitative effect.

It is yet to be assessed what our observations mean when transferred to the human situation Our rat studies did not provide evidence to support the hypothesis that alcohol drinkers'increased risk of cancer is due to enhanced bioactivation of B(*a*)P (IARC, 1988). However, a change in phase II metabolism might play a role. The decrease in epoxide hydrolase activity we measured earlier (van de Wiel *et al*, 1993*)* can be related to the increased amounts of DNA adducts in the liver of ethanol-treated rats reported here.

REFERENCES

Bodell WJ, Devanesan PD, Rogan EG, Cavalieri EL ^{32}P-Postlabeling analysis of benzo(a)pyrene-DNA adducts formed in vitro and in vivo Chem Res Toxicol 2 312-315, 1989

Brauze D, Mikstacka R, Baer-Dubowska W Formation and persistence of benzo(a)pyrene-DNA adducts in different tissues of C57BL/10 and DBA/2 mice Carcinogenesis 12(9) 1607-1611, 1991

Fraumeni JF Epidemiological Opportunities in Alcohol Related Cancer Cancer Res, 39 2851-2852, 1979

Garro AJ, Lieber CS Alcohol and Cancer Annu Rev Pharmacol 30 219-249, 1990

Gupta RC, Reddy MV, Randerath K ^{32}P-Postlabelling of non-radioactive aromatic carcinogen DNA-adducts Carcinogenesis 3 1081-1092, 1982

Gupta RC Enhanced Sensitivity of ^{32}P-Postlabelling Analysis of Aromatic Carcinogen DNA Adducts Cancer Res 45 5656-5662, 1985

Hall M, Grover PL Polycyclic Aromatic Hydrocarbons Metabolism, Activation and Tumor Initiation In Cooper CS, Grover PL "Chemical Carcinogenesis and Mutagenesis I, chapter 9 " Germany Springer-Verlag, 1990

International Agency for Research on Caner "IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Vol 44 Alcohol Drinking" Lyon IARC, 1988

International Agency for Research on Cancer "IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Vol 32 Polynuclear Aromatic Compounds - Part 1 " Lyon IARC, 1983

International Agency for Research on Cancer "IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Vol 38 Tobacco Smoking" Lyon IARC, 1986

Ioannides C, Parke DV The Cytochrome P450 I gene family of microsomal hemoproteins and their role in the metabolic activation of chemicals Drug Metabolism Reviews 22(1) 1-85, 1990

Kriek E, van Schooten FJ, Hillebrand MJX, den Engelse L, van Leeuwen FE DNA-adducten maat voor Blootstelling aan carcinogenen Chemisch Magazine september 517-518, 1989

Lieber CS, Seitz HK, Garro AJ, Worner TM Alcohol Related Diseases and Carcinogenesis Cancer Res 39 2863-2886, 1979

Phillips DH Fifty years of Benzo(a)pyrene Nature 303 486-472, 1983

Phillips DH Modern Methods of DNA Adduct Determination In Cooper CS, Grover PL "Chemical Carcinogenesis and Mutagenesis I, chapter 13 " Germany, Springer-Verlag, 1990

Rothman K, Keller A The effect of joint exposure to alcohol and tobacco on risk of cancer of the mouth and pharynx J Chron Dis 25 711-716, 1972

Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS, Levin W Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid J Biol Chem 260 8385-6393, 1985

Standard Operating Procedure Voorschrift voor de bepaling van benzo(a)pyreen DNA adducten met behulp van de ^{32}P-postlabelling-methode en nuclease P1 behandeling Laboratorium voor Carcinogenese en Mutagenese,RIVM Bilthoven nr LCM/702, 1991

U S Department of Health & Human Services Toxicological Profile for Polycyclic Aromatic Hydrocarbons Clement International Corporation Contract No 205-88-0608

Villa E, Melegari M, Scaglioni PP, Trande P, Cesaro P, Manenti F Hepatocellular carcinoma risk factors other than HBV Ital J Gastroenterol 23 457-460, 1991

van de Wiel JAG, Duijf CMP, Pertijs JCLM, Copius Peereboom-Stegeman JHJ, Bos RP Growth and liver morphology after long-term ethanol consumption of rats Lab Anim 24 265-272, 1990

an de Wiel JAG, Meuwissen M, Kooy H, Fijneman PHS, Noordhoek J, Bos, RP Influence of long-term ethanol treatment on in vitro biotransformation of benzo(*a*)pyrene in microsomes of the liver, lung and small intestine from male and female rats Biochem Pharmacol 44 1977-1984, 1992

van de Wiel JAG, Fijneman PHS, Teeuw KB, van Ommen B, Noordhoek J, Bos RP Influence of long-term ethanol-treatment on rat liver biotransformation enzymes Alcohol *(in press)*

CHAPTER VIII

SUMMARY AND GENERAL DISCUSSION

Epidemiologists estimate that in principle 70% of human cancer would be preventable if the main risk and antirisk factors could be identified (Doll & Peto, 1981). Risk factors can interact, resulting in synergistic or antagonistic effects. Human data show that joint exposure to tobacco smoke and alcoholic beverages significantly increases the relative cancer risk at various sites (General Introduction par. 4.2). This findings suggest that PAHs and ethanol (from tobacco smoke and alcoholic drinks respectively) do not act independently. This thesis is a contribution to the study of such toxicological interactions. Using a rat model, it describes the combined effect of ethanol and B(*a*)P, two of the many carcinogenic chemicals people are exposed to by themselves and others (Group 1 according to the IARC classification, IARC 1983, IARC 1988).

In *Chapter I* human exposure patterns, toxicokinetics and health effects are described for the two compounds individually. Ethanol is mostly consumed voluntarily as part of alcoholic beverages. In the Netherlands, the pure ethanol consumption per citizen increased from 2.6 l per year in 1960 to 8.3 l per year in 1988 (de Zwart, 1989). There are 200000 alcoholics and half a million heavy drinkers who consume 8-12 drinks a day (Schoemaker-Hol, 1988). Moderate alcohol consumption is hard to define, but 17-21 drinks a week is mentioned in several studies (Anderson, 1986; McDonald, 1982).

The enzyme with the highest affinity for ethanol is the alcohol dehydrogenase; it performs the first step in ethanol metabolism (Lieber & DeCarli, 1970). However, ethanol is also converted by the hepatic cytochrome P450 (Rubin *et al*, 1971). An isozyme of cytochrome P450 typically induced by ethanol is P4502E1. Other isozymes of P450 are also influenced. Alcohol consumption is associated with many health problems. There is an association with cancer of the oesophagus, mouth, pharynx, stomach and liver.Tobacco smoking and alcohol drinking are often correlated at the individual level, and tobacco smoke is a cause of cancer at many of the sites also related to alcohol consumption (IARC, 1986).

Polycylic Aromatic Hydrocarbons (PAHs) are important constituents of cigarette smoke. PAHs can be formed by thermal decomposition of any organic materials containing carbon and hydrogen. Thermal decomposition of pit coal, cellulose, tobacco and also of polyethylene and polyvinylchloride which is carried out at 100°C yields very similar PAH profiles (Grimmer, 1983). The routes of environmental exposure to PAHs for the general population are the inhalation of cigarette smoke, and the consumption of water and food. The general population has a relatively low exposure. In occupational situations however, this can be considerably higher. Pathways for exposure of workers to PAHs are the inhalation of vapors or particles, contamination of the skin and ingestion. When PAHs with four or more rings occur in the atmosphere, they are primarily associated with particles. The inhalation of particles may represent an indirect route of ingestion.

The inhaled larger particles will be trapped in the mucous fluids of the upper and middle respiratory system. After transport to the pharynx, they will be swallowed.

Epidemiological studies have demonstrated that smoking of tobacco is causally related to cancer of the respiratory tract, the upper digestive tract, pancreas, renal pelvis, bladder, and cervix (IARC, 1986). Excess of leukaemia, lung and scrotum cancer and of cancer of the gastro-intestinal tract was found in occupationally exposed individuals. Metabolism of PAHs is dominated by oxidation through the microsomal cytochrome P450 dependent monooxygenase system (Nebert & Gonzalez, 1987). Certain 'bay region diol epoxides' are the main DNA binding metabolites of B(*a*)P (Sims et al, 1974). These are held responsible for the initiation of cancer.

Finally *Chapter I* discusses possible levels of interaction between ethanol and PAHs. The compounds can interact during the absorbtion phase, the biotransformation phase and during DNA-adduct formation.

Chapter II deals with the design of a method to administer ethanol at moderate levels to rats during a longer period. A diet is chosen of standard food pellets that are low in fat content and high in carbohydrate. Ethanol is added to the drinking water as the only source of fluid. The ethanol dose had to be gradually increased from 2% to 15% in three weeks. Then the rats stayed for three weeks at a level of 15% ethanol in their drinking water. This method led to a considerable intake of ethanol (6-14 g/kg bw/day) that is 20% of the daily caloric intake. Electron microscopy of the rat livers confirmed proliferation of SER as an effect of ethanol. The livers of ethanol-treated female rats contained more fat droplets than the livers of male rats. No effects on mitochondria nor severe pathological effects such as liver necrosis or cirrhosis were observed. The growth and development of the rats as based on the body weight curves was normal during the treatment. The ratio of liver weight to body weight did not differ significantly between ethanol-treated and control rats. Human alcoholics are defined as those who are chronically consuming at least 20% of their daily caloric intake as ethanol (Lelbach, 1974). This rat model therefore seems realistic. We used this model for further studies on the effects of long-term ethanol exposure on the toxicokinetics and DNA adduct formation of B(*a*)P.

Chapter III excretion of B(*a*)P and metabolites in urine and feces of rats is described. The influence of the route of administration, sex and long-term ethanol treatment was studied. The administration route largely determined the total amount of excreted metabolites. Both after oral (p.o.) and intraperitoneal (i.p.) treatment, much more metabolites were found in feces compared to urine. Besides the relatively high fecal excretion of unmetabolized B(*a*)P, metabolites and mutagenic products in feces were much higher after p.o. compared to i.p. B(*a*)P administration. The rate of B(*a*)P-(metabolite) excretion was higher as well.

Males had a higher urinary excretion of mutagenic compounds as well as of phenolic metabolites than females. In feces, male rats excreted a higher percentage of B(*a*)P as metabolites and as mutagenic products than female rats.

In the four groups investigated (female i.p., male i.p., female p.o., male p.o.) ethanol treatment caused a lowered 3-hydroxy-B(*a*)P excretion in feces. In the urine of female rats that received an i.p. dose of B(*a*)P, the ethanol treatment resulted in a statistically significant reduction of 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P.

In this study the influence of sex and administration route on the excretion of B(*a*)P metabolites was more pronounced than the effect of ethanol treatment.

A reduced amount of B(*a*)P metabolites in urine and/or feces after ethanol treatment could be due to changes in absorption, biotransformation or excretion. Possible changes in biotransformation were further investigated as described in *Chapter IV.* The influence of long-term ethanol treatment on *in vitro* biotransformation of B(*a*)P was studied in microsomal fractions of liver, lung and small intestine from male and female rats. Quantitatively, the most important metabolite formed in all organs, 3-hydroxy-B(*a*)P, accounted for 70-85% of all metabolites measured. This was in accordance with our *in vivo* studies. In intestinal microsomes, 9-hydroxy-B(*a*)P was the second important metabolite. In lung microsomes it was B(*a*)P-7,8-diol and in liver microsomes B(*a*)P-9,10-diol. There were large differences in microsomal activity between the three organs. In liver samples nmoles of B(*a*)P were metabolized per mg protein per min, in lung samples and samples of small intestine pmoles of B(*a*)P were metabolized per mg protein per min. The sex difference concerning B(*a*)P metabolism, found in *in vivo* studies proved organ dependent in *in vitro* studies. In liver microsomal fractions of male rats we found a 2-5 times higher formation of diols and phenols compared to females. However, no sex difference in lung microsomes and a small sex difference in microsomes of the small intestine were observed. Direct evidence for the participation of the male specific isozyme in B(*a*)P biotransformation in liver was obtained using an antibody to P4502C11/C6. We were able to inhibit about 20-80% of the microsomal B(*a*)P metabolism, depending on the metabolite measured. Apparently, P4502C11/C6 plays an important role in B(*a*)P biotransformation in uninduced, as well as in ethanol-treated male rats.

The effect of the ethanol treatment was different in the three organs. In liver both 9- and 3-hydroxylation of B(*a*)P was significantly decreased. For other P450 inducers like phenobarbital, 3-methylcholanthrene or PCB's it is known that they leave the sex-specific P450 isoenzymes unaffected or depress them (Wortelboer, 1991). The latter possibility seems also to hold for ethanol. When using antibodies to P4502C11\6, the percentage remaining activity of B(*a*)P metabolite formation was significantly higher in ethanol-treated, compared to control rats. This confirmed the down-regulating influence of ethanol on sex

specific P450 isoenzyme activity and consequently on B(*a*)P biotransformation. In the lung also, a tendency for decrease in B(*a*)P metabolism was observed after ethanol treatment. In the small intestine, however, a significant increase in the formation of B(*a*)P phenol metabolites was seen (+50% males, +20% females) after ethanol treatment.

To study the observed effect of ethanol treatment on the biotransformation even further, several different enzyme activities were measured in liver microsomes of male and female rats (*Chapter V*). A significant induction of P450 and aniline 4-hydroxylase activity (2E1) was found. EROD activity was not changed after ethanol treatment. This activity is associated with P4501A1, so there are no indications for a change in P4501A1 activity. When several doses of B(*a*)P were given, with or without ethanol, the self-inducing effect of B(*a*)P was clear and EROD activity increased with a factor 6. PROD (P4502B1/2B2) was not changed by ethanol treatment. Testosterone metabolism was differently influenced by ethanol treatment. A clear decrease of 25% in activity was measured for 6ß-hydroxytestosterone in males. Formation of this metabolite represents the activity of the male specific P450's 3A2 and 2C13. In females 12ß-hydroxytestosterone formation rate was lowered to 50% of the initial value (unknown P450). The constitutive sex specific enzymes in males and females are probably responsible for most of the B(*a*)P biotransformation activity in non-induced rats (Kato *et al.*, 1986). Sex differences in testosterone metabolism were described by Kobliakov (1991). He found 16α-hydroxytestosterone and 6ß-hydroxytestosterone formation to be higher in males. In livers of male rats, we found 5 of the 9 testosteron metabolites formed at higher level than in livers of females. Among them 16α- and 6β–hydroxytestosterone. Two metabolites were formed at a lower level in male than in female rats.

The earlier observed decreases in excretion (*Chapter III*) and in *in vitro* formation (*Chapter IV*) of B(*a*)P metabolites in ethanol-treated rats can be linked to the enzyme measurements (*Chapter V*). B(*a*)P metabolism was largely dependent on the sex specific P450s as shown by inhibition with the P4502C11/2C6 antibody. After ethanol treatment P4502C11/C6 dependent B(*a*)P metabolism was decreased. In line with this we expected a reduction of 16α- and 2α-hydroxytestosterone after ethanol treatment since these are metabolites specially formed by P4502C11. However, a lowered *in vitro* formation of the 6β–testosterone metabolite was observed after ethanol treatment. This indicates a reduction of another sex specific P450: P4502C13/3A2.

The phase II enzymes that are important in B(*a*)P biotransformation, glutathione S-transferase and epoxide hydrolase, were both influenced by ethanol treatment. In our measurements the activity of GST was not significantly changed. The amount of subunit 1 however, was significantly decreased in males and females. The pie-family (GST subunits 7-7) was described to be the most

important enzyme in conjugating anti-BPDE and other electrophilic metabolites of benz(*a*)anthracene and chrysene (Jernström, 1989). In that case a reduction in subunit 1 should have no great impact on the amount of anti-BPDE conjugated. Epoxide hydrolase is important in the formation of diols from B(*a*)P-epoxides and in the formation of tetrols from B(*a*)P-dihydrodiolepoxides. Epoxide hydrolase activity was decreased considerably by ethanol treatment. In *in vivo* and *in vitro* studies we observed decreased levels of B(*a*)P phenolic as well as diolic metabolites. In a study of Selkirk (1975) inhibition of epoxide hydrolase was correlated with an increase of phenol metabolites, while diols disappeared. According to that study the decrease in epoxide hydrolase activity could not be the source of a decreased formation of both phenols and diols. However, it is conceivable that the decrease in epoxide hydrolase activity we measured could have an effect on the amount of the reactive anti-BPDE formed from the B(*a*)P 7,8-diol 9,10 epoxide.

In *Chapters VI* and *VII*, the influence of long-term ethanol treatment on B(*a*)P DNA-adduct formation is described. We measured DNA-adduct formation in liver and intestinal epithelial cells of rats after one or several doses of B(*a*)P.

Ethanol treatment affected the level of certain DNA adducts in liver and intestine. A significant enhancement was found for the main adduct in liver after ethanol treatment. In the gut, ethanol treatment tended to have the opposite effect. Possibly, ethanol treatment affects gut epithelial tissue in such a way that it becomes more permeable for non-metabolized B(*a*)P (Persson, 1991). This would give rise to a lower adduct level in the intestinal epithelium, but at the same time the amount of B(*a*)P that reaches the liver may be increased and consequently more reactive metabolites could be formed there.

If the results are combined with our earlier studies a lowered formation of B(*a*)P phenol metabolites in liver microsomes is correlated with an enhanced DNA-adduct formation. In small intestine we measured an increase of the formation of phenolic metabolites and a decreased DNA-adduct level.

For compounds like nitrosamines there was experimental evidence that alcohol could act as a promoter at metabolic level by inducing the biotransformation enzymes that turn a procarcinogen into a carcinogen. However, this is true for compounds like nitrosamines and small organocyclic molecules that are substrate for the ethanol inducible P4502E1. B(*a*)P is no substrate for this isoenzym, as was described in *Chapter IV*, so the enhanced formation of B(*a*)P-DNA adducts in liver cannot be explained this way.

Ethanol can influence the enzymes that deactivate a reactive intermediate, like epoxide hydrolase. In the liver this enzyme is lowered after ethanol treatment. It is conceivable that this effect enhanced the B(*a*)P-DNA adduct level formation in the liver as was measured.

absorption distribution elimination	phenol and diol metabolites in urine and feces -phenol and diol metabolites of B(a)P lowered after EtOH	chapter III
metabolism	microsomes of liver -lowered formation of B(a)P phenols after EtOH microsomes of lung -lowered formation of phenols after EtOH microsomes of small intestine -enhanced formation of phenols after EtOH	chapter IV
	with anti P4502C11/2C6 21-83% inhibition of B(a)P metabolism depending on the metabolite measured	chapter IV
	enzymes in liver microsomes -Phase I P450 total enhanced after EtOH P4502E1 (An 4-H) enhanced after EtOH P4501A1 (EROD) unchanged after EtOH P4502B1/2B2 (PROD) unchanged after EtOH P4503A2/2C13 (6ß-OHT) lowered (*) after EtOH P450 ? (12ß-OHT) lowered (**) after EtOH P4502C11 (16α-OHT) unchanged (*) after EtOH P4502C11 (2α-OHT) unchanged (*) after EtOH P4502C6 ? -Phase II glutathion S-transferase unchanged after EtOH microsomal epoxide hydrolase lowered after EtOH	chapter V
effect	DNA adducts after EtOH -in liver enhanced -lung enhanced -small intestine lowered	chapter VI,VII

Table 1 Summary of ethanol and B(*a*)P interaction levels and findings in the present study
* in male rats, ** in female rats

Adduct levels are a result of formation and breakdown. Breakdown of adducts is caused by DNA repair mechanisms, necrosis of cells and cell turnover. Alcohol can inhibit DNA repair and influence sister chromatid exchanges, exerting a promoting activity (Obe & Ristow, 1979; Garro, 1986). This effect could be organ dependent.

Epithelial cells of the gastro-intestinal tract have a relatively high turnover. The faster decline in adduct levels in the small intestine of ethanol-treated rats could be partly explained by the fact that this turnover is even further enforced by ethanol. DNA adducts can be easily diluted in this way. In contrast, DNA-adduct are better conserved in the liver with its low cell turnover.

The results of our studies are comprised in table 1.

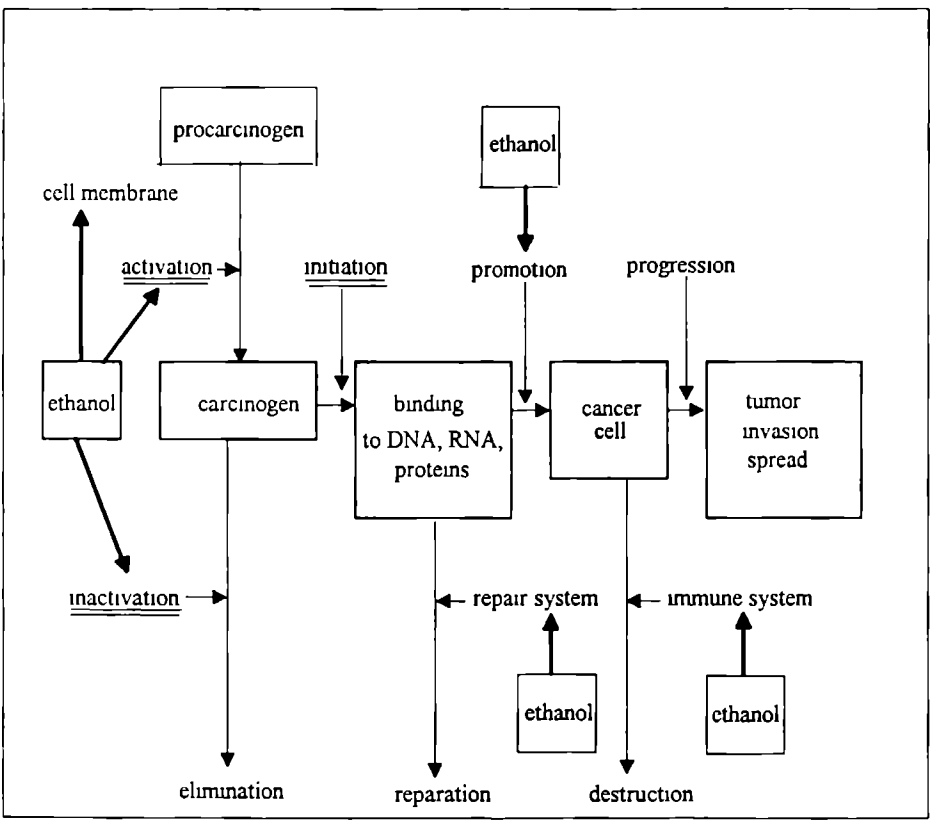


Figure 1 Scheme of two-step carcinogenesis and possible sites of action of ethanol (Seitz, 1988) Double underlined interaction sites were the subject of this study

Table 1 is an extension of table 3 of paragraph 5 of the *General Introduction.*

What our observations mean when transferred to the *human situation* is yet to be assessed. On the one hand, our rat studies did not provide evidence to support the hypothesis (IARC, 1988) that alcohol drinkers' increased risk of cancer is due to enhanced bioactivation of B(*a*)P. On the other hand, we did observe increased amounts of DNA-adducts in rat livers. There might well be a causal relationship

We studied the influence of ethanol treatment on activation and inactivation of the carcinogen B(*a*)P by measuring excretion products, metabolic activity in several organs and DNA adducts. Not all possible levels of interaction between ethanol and B(*a*)P were measured Some more sites are shown in figure 1 (identical to figure 6 in the *General Introduction*).

with the measured change in phase II metabolism, the decrease in epoxide hydrolase activity. Many different techniques and samples, also of human origin, will have to be used in *in vivo* as well as in *in vitro* studies to elucidate further the epidemiological finding of the synergistic effect of ethanol consumption and tobacco smoking on carcinogenesis

REFERENCES

Anderson P What is a safe level of alcohol consumption? Stress Med 1986,2 99-101

Doll R & Peto R The causes of cancer Oxford Oxford Univ Press, 1981

Garro AJ, Espina N, Farinati F, Lieber CS Ethanol and the repair of deoxyribonucleic acid (DNA) Alcohol Health Res World 1986,10(3) 26-7

Grimmer G Environmental Carcinogens Polycyclic Aromatic Hydrocarbons Boca Raton CRC Press, 1983

IARC Polynuclear Aromatic Compounds Part1, Chemical, Environmental and Experimental Data Lyon IARC, 1983 (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans)

IARC Alcohol drinking Lyon IARC, 1988 (IARC Monographs on the evaluation of carcinogenic risks to humans, Vol 44)

IARC Tobacco smoking Lyon IARC, 1986 (IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 38)

Jernstrom B, Dock L, Hall M, Mannervik B, Tahir MK, Grover PL Glutathione transferase catalyzed conjugation of benzo(a)pyrene 7,8-diol 9,10-epoxide with glutathione in human skin Chem-Biol Interactions 70,173-180 1989

Kato R, Yamazoe Y, Shimada M, Murayama N, Kamataki T Effect of growth hormone and ectopic transplantation of pituitary gland on sex-specific forms of cytochrome P450 and testosterone and drug oxidations in rat liver J Biochem 100,895-902 1986

Kobliakov V, Popova N, Rossi L Regulation of the expression of the sex-specific isoforms of cytochrome P-450 in rat liver Eur J Biochem 195,585-591 1991

Lelbach WK In Gibbins-RJ, Israel-Y, Kalant-H, Propham-RE, Schmidt-W & Smart-RG, eds Research Advances in Alcohol and Drug Problems New York John Wiley, 1974 1, 93

Lieber CS & DeCarli LM Hepatic microsomal ethanol-oxidizing system in vitro characteristics and adaptive properties *in vivo* J biol Chem 1970,245 2505-2512

McDonald J Moderate amounts of alcoholic beverages and clinical nutrition J Nutr Educ 1982 14,58-60

Nebert DW & Gonzalez FJ P-450 genes structure, evolution and regulation Ann Rev Biochem 1987,56 945-993

Obe G & Ristow H Mutagenic, carcinogenic and teratogenic effects of alcohol Mutation Res 1979,65 229-59

Persson J, Berg NO, Sjölund K, Stenling R, Magnusson PH Morphologic changes in the small intestine after chronic alcohol consumption Scand J Gastroenterol 1990,25 173-84

Rubin E, Gang H, Lieber CS Interaction of ethanol and pyrazole with hepatic microsomes Biochem Biophys Res Commun 1971,42 1-8

Schoemaker-Hol MAM Sociaal drinken is asociaal Intermediair 1988 24(27),47-51

Seitz HK, Simanowski UA Alcohol and carcinogenesis Ann Rev Nutr 1988,8 99-119

Selkirk JK, Croy RG, Gelboin HV Isolation by high pressure liquid chromatography and characterization of benzo(a)pyrene-4,5-epoxide as a metabolite of benzo(a)pyrene Arch Biochem Biophys 168 322-326,1975

Sims P, Grover PL, Swaisland A, Pak K & Hewer A Metabolic activation of benzo(a)pyrene proceeds by a diolepoxide Nature 1974,252 326-328

Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J & Blaauboer BJ Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures Biochem Pharmacol 1991,42(2) 381-90

de Zwart WM Alcohol, tabak en drugs in cijfers Utrecht Nederlands Instituut voor Alcohol en Drugs, 1989

SAMENVATTING EN ALGEMENE DISCUSSIE

Epidemiologen schatten dat in principe kanker bij de mens voor 70% te voorkomen zal zijn als de belangrijkste risico- en anti-risicofactoren geïdentificeerd kunnen worden (Doll & Peto, 1981) Risicofactoren kunnen elkaar beïnvloeden, hetgeen kan resulteren in synergistische of antagonistische effecten Uit onderzoek bij mensen is gebleken dat gecombineerde blootstelling aan tabaksrook en alcoholhoudende dranken het risico op kanker voor verscheidene organen verhoogt (General Introduction, par 4 2) Deze bevindingen suggereren dat polycyclische aromatische koolwaterstoffen (PAKs) en ethanol (respectievelijk bestanddelen van tabaksrook en alcoholhoudende dranken) niet onafhankelijk van elkaar hun effect hebben Dit proefschrift is een bijdrage aan het bestuderen van zulke toxicologische interacties Aan de hand van een proefdiermodel wordt het gecombineerde effect van ethanol en benzo(*a*)pyreen [B(*a*)P] beschreven Ethanol en B(*a*)P zijn twee voorbeelden van kankerverwekkende stoffen (classificatie in groep 1 volgens de IARC (IARC 1983, 1989) waaraan mensen zichzelf blootstellen en worden blootgesteld

In *Hoofdstuk 1* worden blootstellingspatronen, toxicokinetiek en gezondheidseffecten van beide verbindingen afzonderlijk beschreven Ethanol wordt vrijwel altijd vrijwillig geconsumeerd als bestanddeel van alcoholhoudende dranken In Nederland steeg de ethanolconsumptie per persoon van 2,6 liter per jaar in 1960 tot 8,3 liter per jaar in 1988 (de Zwart, 1989) Er zijn in ons land 200 000 alcoholisten en een half miljoen 'zware drinkers', die 8-12 glazen per dag consumeren (Schoenmaker-Hol, 1988) 'Gematigde alcoholconsumptie' is moeilijk te definieren, volgens verschillende auteurs is hiervan sprake bij een gebruik van 17-21 glazen per week (Anderson, 1986, McDonald, 1982)

Het enzym met de hoogste affiniteit voor ethanol is het alcoholdehydrogenase, het is betrokken bij de eerste stap van het ethanol-metabolisme (Lieber & DeCarli, 1970) Ethanol wordt ook omgezet door het enzymcomplex cytochroom P450 (Rubin *et al*, 1971) Een iso-enzym van cytochroom P450 dat specifiek door ethanol wordt geïnduceerd is het P4502E1 Ook andere iso-enzymen van P450 ondervinden deze invloed

Alcoholconsumptie is geassocieerd met uiteenlopende gezondheidsproblemen Er is een correlatie met slokdarm-, mond-, keel-, maag-, en leverkanker Het roken van tabak en het drinken van alcohol komen vaak samen voor Er is ook een correlatie tussen het roken van tabak en verschillende van de zojuist genoemde soorten kanker (IARC, 1986)

Polycylische aromatische koolwaterstoffen (PAKs) zijn belangrijke bestanddelen van sigarettenrook PAKs kunnen ontstaan bij thermische ontleding

van organisch materiaal dat koolstof en waterstof bevat. Thermische ontleding van steenkool, cellulose, tabak en ook van polyethyleen en polyvinylchloride bij 100 °C geeft sterk op elkaar lijkende PAK-profielen (Grimmer, 1983).

De blootstellingsroutes van de algemene bevolking aan PAKs lopen via het inhaleren van sigarettenrook en de consumptie van water en voedsel. De algemene bevolking heeft een relatief lage expositie. In arbeidssituaties kan de blootstelling veel hoger zijn. Blootstellingsroutes voor werknemers aan PAKs zijn de inademing van dampen of deeltjes, opname via de huid en inslikken. Als PAKs met vier of meer aromatische ringen in de lucht komen, zijn ze vooral geassocieerd met deeltjes. De geïnhaleerde deeltjes worden door het slijm van het boven- en middendeel van het ademhalingssysteem ingevangen. Via transport naar de keelholte worden ze dan ingeslikt.

Epidemiologisch onderzoek heeft aangetoond dat het roken van tabak oorzakelijk verbonden is met kanker van de ademhalingsorganen, het bovenste deel van het spijsverteringskanaal, de alvleesklier, nierbekken en baarmoederhals (IARC, 1986). Bij beroepsmatig blootgestelde mensen is een overmaat aan leukemie, long- en scrotumkanker en kanker van het gastro-intestinale stelsel gevonden. Het metabolisme van PAKs vindt voornamelijk plaats via oxidatie door het cytochroom P450 afhankelijke mono-oxygenase systeem (Nebert & Gonzalez, 1987). Bepaalde 'bay region' diol-epoxides zijn de belangrijkste DNA-bindende metabolieten van B(*a*)P (Sims *et al.*, 1974). Deze worden verantwoordelijk geacht voor de initiatie van kanker.

Ten slotte worden in *Hoofdstuk I* mogelijkheden besproken van interactie tussen ethanol en PAKs: deze kan plaatsvinden in de absorptiefase, de biotransformatiefase en bij de DNA-adductvorming.

Hoofdstuk II gaat over het ontwikkelen van een methode om ratten gedurende langere tijd aan matige hoeveelheden ethanol bloot te stellen via hun dagelijkse voeding. Er werd een dieet gekozen van standaard voedselpellets die weinig vet en veel koolhydraat bevatten, in combinatie met ethanol in het drinkwater. Omdat ratten moeten wennen aan de ethanol, werd de concentratie in drie weken geleidelijk opgevoerd van 2% tot 15%. Vervolgens kregen de ratten drie weken 15% ethanol in hun drinkwater. Deze methode leidt tot een ethanolopname van 6-14 g/kg lichaamsgewicht/dag. Dit is 20% van de dagelijkse calorie-opname. Alcoholisten worden gedefinieerd als diegenen die chronisch ten minste 20% van hun dagelijkse calorieën opnemen in de vorm van ethanol (Lelbach, 1974). Dit proefdiermodel lijkt in dit opzicht een hoge blootstelling te realiseren. De resulterende bloedalcohol-waarden zijn echter niet zo hoog.

Electronenmicroscopische opnames van de rattelevers bevestigden proliferatie van het glad endoplasmatisch reticulum tengevolge van ethanolblootstelling. De levers van aan ethanol blootgestelde vrouwtjesratten bevatten meer vetdruppels dan die van de gelijkelijk behandelde mannetjesratten.

Er werden geen effecten gevonden op de mitochondria, noch pathologische effecten als levernecrose en levercirrhose. De groei van de ratten was blijkens de lichaamsgewichtcurves, tijdens de behandeling normaal. De verhouding levergewicht/lichaamsgewicht bij de met ethanol behandelde ratten was niet significant verschillend van die bij de onbehandelde.

We gebruiken dit model voor verdere studies naar de effecten van langdurige ethanol blootstelling op de toxicokinetiek en DNA-adductvorming van B(*a*)P.

In *Hoofdstuk III* wordt de excretie van B(*a*)P en metabolieten in urine en faeces van ratten beschreven. De invloed van de toedieningsweg van B(*a*)P, het geslacht van de ratten en het effect van langdurige ethanolblootstelling is bestudeerd. De toedieningsweg bepaalde in belangrijke mate de totale uitgescheiden hoeveelheid B(*a*)P (metabolieten). Zowel na orale (p.o.) als na intraperitoneale (i.p.) toediening, werden veel meer metabolieten gevonden in de faeces dan in de urine. Naast de relatief grote hoeveelheid faecaal uitgescheiden ongemetaboliseerd B(*a*)P, was de hoeveelheid faecale metabolieten en mutagene producten veel hoger na p.o. toediening van B(*a*)P, dan na i.p. toediening. De snelheid van B(*a*)P (metaboliet)uitscheiding was ook hoger na p.o. toediening.

Mannetjesratten vertoonden een grotere urinaire uitscheiding van mutagene componenten en fenol metabolieten dan vrouwtjesratten.

In de vier onderzochte groepen (vrouwtjes i.p., mannetjes i.p., vrouwtjes p.o., mannetjes p.o., van elke groep kreeg de helft ethanol) veroorzaakte de toediening van ethanol een verlaagde uitscheiding van 3-hydroxy-B(*a*)P in de faeces. Bij vrouwtjesratten die een i.p. dosis B(*a*)P kregen toegediend, resulteerde de ethanolbehandeling in een statistisch significante afname van de uitscheiding van 9-hydroxy-B(*a*)P en 3-hydroxy-B(*a*)P in de urine.

In dit onderzoek bleken de invloed van het geslacht en die van de toedieningsweg van B(*a*)P meer uitgesproken te zijn op de uitscheiding van B(*a*)P-metabolieten dan het effect van de ethanolbehandeling.

De verminderde hoeveelheid B(*a*)P-metabolieten in urine of faeces na ethanolbehandeling kan het gevolg zijn van veranderingen in absorptie, biotransformatie of excretie. Mogelijke veranderingen in biotransformatie zijn verder onderzocht zoals beschreven in *hoofdstuk IV*. De invloed van langdurige blootstelling aan ethanol op de *in vitro* biotransformatie van B(*a*)P werd bestudeerd in enzymbevattende orgaanfracties van lever, longen en dunne darm van mannetjes- en vrouwtjesratten. De, in alle organen gevormde, kwantitatief belangrijkste metaboliet 3-hydroxy-B(*a*)P maakte 70-85% van de totale hoeveelheid gemeten metabolieten uit. Dit stemde overeen met de bevindingen uit ons *in vivo* onderzoek. In de dunne-darmfracties was 9-hydroxy-B(*a*)P de tweede belangrijke metaboliet. In longfracties was het B(*a*)P-7,8-dihydrodiol en in

leverfracties B(*a*)P-9,10-dihydrodiol. Er waren grote verschillen in metabole activiteit tussen de fracties van de drie organen. Het sexe-verschil in B(*a*)P-metabolisme, gevonden in het *in vivo* onderzoek, bleek bij *in vitro* onderzoek orgaanafhankelijk te zijn. In leverfracties van mannetjesratten was de vorming van diolen en fenolen 2 tot 5 keer zo hoog als bij de vrouwtjesratten. Er is echter geen geslachtsverschil gevonden in metabole activiteit van longfracties, en slechts een klein geslachtsverschil in die van dunne-darmfracties.

Een direct bewijs voor de betrokkenheid van het 'male-specific' iso-enzym van P450 bij B(*a*)P-biotransformatie in de lever werd verkregen met behulp van het antilichaam van P4502C11/C6. We waren in staat 20-80% van het B(*a*)P-metabolisme te remmen, afhankelijk van de gemeten metaboliet. Blijkbaar speelt P4502C11/C6 een belangrijke rol in de B(*a*)P-biotransformatie bij zowel ongeïnduceerde als aan ethanol blootgestelde mannetjesratten.

Het effect van de blootstelling aan ethanol was verschillend in de drie organen. In de leverfracties nam de 3- en 9-hydroxylering van B(*a*)P significant af. Van verschillende 'klassieke' P450-inducers, zoals fenobarbital, 3-methylcholanthreen of PCBs, is bekend dat ze de sexe-specifieke P450-iso-enzymen hetzij niet beïnvloeden hetzij remmen (Wortelboer, 1991). Die laatste mogelijkheid lijkt op te gaan voor ethanol. Als antilichamen voor P4502C11/C6 werden gebruikt, was het percentage overgebleven activiteit (B(*a*)P-metabolietvorming) significant hoger in met ethanol behandelde ratten, vergeleken met controleratten. Dit bevestigde de mogelijke 'down-regulatie' van ethanol op de sexe-specifieke iso-enzymactiviteit van P450 en daaraan gekoppelde B(*a*)P-biotransformatie. In longfracties werd een tendens tot verlaging van B(*a*)P-metabolisme waargenomen na ethanolbehandeling. In dunne-darmfracties werd daarentegen een significante toename van de vorming van B(*a*)P-fenol metabolieten gemeten (+50% in de mannetjesraten, +20% in de vrouwtjesratten) na ethanolbehandeling.

Om het effect van de ethanolbehandeling op de biotransformatie verder te bestuderen werden verschillende enzymactiviteiten gemeten in leverfracties van mannetjes- en vrouwtjesratten. (*Hoofdstuk V*). Er werd een significante inductie van P450- en aniline-4-hydroxylase-activiteit (P4502E1) gevonden. De activiteit van ethoxy-resorufine-O-deethylase (EROD) veranderde niet. Deze activiteit is geassocieerd met P4501A1, dus er is geen indicatie voor een verandering in P4501A1-activiteit. Als meerdere doses B(*a*)P worden gegeven, met of zonder ethanol, is het zelf-inducerende effect van B(*a*)P duidelijk door het toenemen van de EROD activiteit met een factor 6.

Pentoxy-resorufine-O-deethylase-activiteit (PROD) (P4502B1/1B2) werd niet beïnvloed door ethanolbehandeling. Het testosteronmetabolisme werd gedifferentieerd beïnvloed door ethanolbehandeling. Een duidelijke daling van 25% in de vorming van 6β–hydroxytestosteron werd gemeten in leverfracties van

mannetjesratten. Vorming van deze metaboliet representeert de activiteit van de 'male specific' P450-isoenzymen P4503A2 en P4502C13. In vrouwtjesratten was de 12β–hydroxytestosteronvorming verlaagd tot 50% van de oorspronkelijke waarde (onbekend iso-enzym). De constitutief aanwezige geslachtspecifieke P450-iso-enzymen in mannetjes- en vrouwtjesratten zijn waarschijnlijk verantwoordelijk voor het grootste deel van de B(*a*)P-biotransformatieactiviteit in niet-geïnduceerde ratten (Kato *et al.*, 1986). Sexeverschillen in testosteronmetabolisme zijn beschreven door o.a. Kobliakov (1991). Hij vond in mannetjesratten meer vorming van 16α-hydroxytestosteron en 6β-hydroxytestosteron per tijdseenheid. Wij vonden dat vijf van de negen testosteronmetabolieten (waaronder 16α en 6β) in grotere hoeveelheden gevormd worden in levers van mannetjesratten als in levers van vrouwtjesratten. Voor twee metabolieten gold het omgekeerde.

De eerder waargenomen vermindering in de uitscheiding (*Hoofdstuk III*) en in *in vitro* vorming (*Hoofdstuk IV*) van B(*a*)P-metabolieten in aan ethanol blootgestelde ratten kan gecorreleerd worden met de enzymmetingen (*Hoofdstuk V*). B(*a*)P- metabolisme is grotendeels afhankelijk van de sexe-specifieke P450s, zoals aangetoond door inhibitie met het P4502C11/2C6-antilichaam. Na ethanolbehandeling was het P4502C11/2C6-afhankelijke B(*a*)P-metabolisme verlaagd. In lijn hiermee verwachtten we een verminderde vorming van 16α-hydroxytestosteron en 2α-hydroxytestosteron na toediening van ethanol, aangezien dit de testosteronmetabolieten zijn die met name door P4502C11 gevormd zouden worden. Echter, gemeten werd een verlaagde *in vitro* vorming van de testosteronmetaboliet 6β–hydroxytestosteron. Dit is een indicatie voor de reductie van een ander sexe-specifiek P450: P4502C13/3A2.

De fase II-enzymen die belangrijk zijn voor de biotransformatie van B(*a*)P, glutathion-S-transferase en epoxide-hydrolase, werden beide beïnvloed door ethanol. Blijkens onze metingen was de activiteit van glutathion-S-transferase niet veranderd, maar de hoeveelheid subunit 1 was in mannetjes- en vrouwtjesratten significant verlaagd. De pie-familie (GST subunits 7-7) is beschreven als het belangrijkste iso-enzym in het conjugeren van *anti*-BPDE en andere electrofiele metabolieten van stoffen als benz(*a*)anthraceen en chryseen (Jernström, 1989). In dat geval zou een afname in subunit 1 (alpha-familie) geen grote invloed hebben op de geconjugeerde hoeveelheid *anti*-BPDE.

Epoxide-hydrolase is belangrijk voor twee stappen van het B(*a*)P-metabolisme; de vorming van diolen uit B(*a*)P-epoxides en de vorming van tetrolen uit B(*a*)P-dihydrodiol-epoxides. De epoxide-hydrolase-activiteit werd aanzienlijk verlaagd door toediening van ethanol. In *in vivo* en *in vitro* onderzoek hebben wij verlaagde hoeveelheden van fenol- en diol-metabolieten van B(*a*)P gemeten. Het is voorstelbaar dat verlaging van de epoxide-hydrolase-activiteit een effect heeft op de hoeveelheid reactief *anti*-BPDE dat uit het B(*a*)P 7,8-diol 9,10-epoxide wordt gevormd.

In *Hoofdstuk VI* en *Hoofdstuk VII* wordt de invloed van ethanol op B(*a*)P-DNA-adductvorming beschreven. We maten DNA-adductvorming in lever en dunne darmcellen van ratten na een of verscheidene doses B(*a*)P.

Behandeling met ethanol beïnvloedde de hoeveelheid van bepaalde DNA-adducten in lever en dunne darm. Er is een significante verhoging van het belangrijkste leveradduct gevonden. In de darm leidde de ethanoltoediening tot een tegengesteld effect. Mogelijk beïnvloedt ethanol darmepitheelweefsel zodanig dat het beter doorlaatbaar wordt voor niet-gemetaboliseerd B(*a*)P (Persson, 1991). Dit zou kunnen leiden tot een lager adduct-niveau in het dunne-darmepitheel terwijl dan de hoeveelheid B(*a*)P die de lever bereikt, verhoogd zal zijn, waardoor hier meer reactieve metabolieten gevormd kunnen worden.

Uit combinatie van de in *Hoofdstuk IV, VI en VII* beschreven resultaten blijkt dat een verlaagde vorming van B(*a*)P-fenol-metabolieten in leverfracties samengaat met een toegenomen DNA-adductvorming. In dunne-darmfracties werd een toename van de vorming van B(*a*)P-fenol metabolieten en een verlaagd DNA-adductniveau gemeten.

Voor verbindingen als nitrosamines is er experimenteel bewijs dat ethanol als een promotor kan werken op het metabole niveau. De biotransformatie-enzymen die het procarcinogeen omzetten in een carcinogeen worden geïnduceerd. Dit geldt echter alleen voor verbindingen als nitrosamines en kleine organocyclische moleculen die substraat zijn voor het door ethanol induceerbare P4502E1. B(*a*)P is geen substraat voor dit iso-enzym, zoals beschreven in *Hoofdstuk IV,* dus de toegenomen vorming van B(*a*)P-DNA-adducten in de lever kan niet via het mechanisme van verhoogde biotransformatie verklaard worden. Ook de verminderde fenolvorming duidt eerder op een verlaagde fase I biotransformatie.

Ethanol kan de enzymen beïnvloeden die reactieve intermediairen de-activeren, zoals epoxide-hydrolase. In de lever was dit enzym verlaagd na ethanolbehandeling. Het is voorstelbaar dat dit effect leidt tot het hogere B(*a*)P-DNA adductniveau zoals door ons werd gemeten.

Adductniveaus zijn een resultaat van vorming en afbraak. Afbraak van adducten wordt veroorzaakt door DNA-reparatiemechanismen, afsterven van cellen en celdeling. Ethanol kan DNA-reparatie inhiberen en sister chromatid exchanges beïnvloeden, waardoor een promoverende werking ontstaat (Obe & Ristow, 1979; Garro, 1986). Dit zou orgaanspecifiek kunnen werken.

Epitheliale cellen van het gastro-intestinale apparaat hebben een relatief hoge turnoversnelheid. De snellere daling van adductniveaus in de dunne darm van met ethanol behandelde ratten kan gedeeltelijk verklaard worden door het feit dat deze turnover nog gestimuleerd wordt door ethanol. DNA-adducten kunnen zo gemakkelijk 'verdund' worden. In de lever worden DNA-adducten beter geconserveerd door de lage celturnover in dit orgaan.

absorptie verdeling eliminatie	fenol and diol metabolieten in urine and faeces -fenol and diol metabolieten van B(a)P verlaagd na EtOH	hoofdstuk III
metabolisme	enzymhoudende fracties van de lever -verlaagde vorming van B(a)P fenolen na EtOH enzymhoudende fracties van de long -verlaagde vorming van fenolen na EtOH enzymhoudende fracties van de dunne darm -verhoogde vorming van fenolen na EtOH	hoofdstuk IV
	met anti-lichaam P4502C11/2C6 21-83% inhibitie van B(a)P-metabolisme, afhankelijk van de gemeten metaboliet	hoofdstuk IV
	enzymen in leverfracties -Fase I P450 totaal toegenomen na EtOH P4502E1 (An4-H) toegenomen na EtOH P4501A1 (EROD) onveranderd na EtOH P4502B1/2B2 (PROD) onveranderd na EtOH P4503A2/2C13 (6ß-OHT) verlaagd (*) na EtOH P450 ? (12ß-OHT) verlaagd (**) na EtOH P4502C11 (16αOHT) onveranderd (*) na EtOH P4502C11 (2α-OHT) onveranderd (*) na EtOH P4502C6 ? -Fase II glutathion-S-transferase onveranderd na EtOH microsomaal epoxide-hydrolase verlaagd na EtOH	hoofdstuk V
effect	DNA-adducten na EtOH -in lever toegenomen -long toegenomen -dunne darm verlaagd	hoofdstuk VI,VII

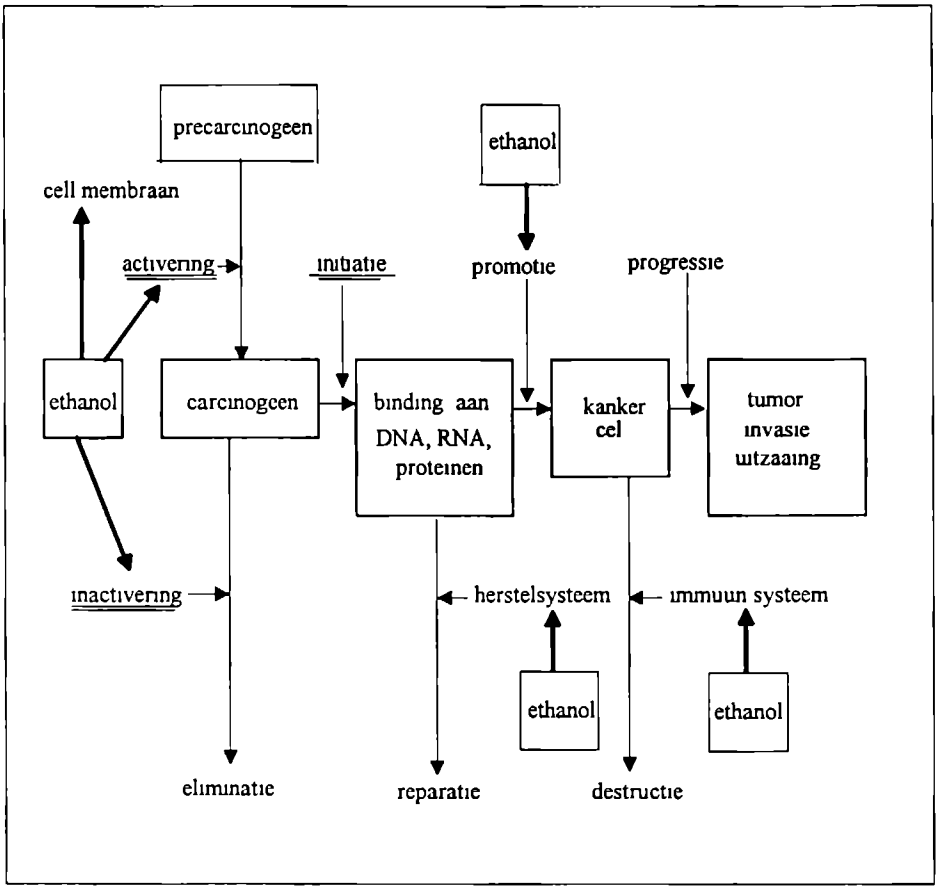
Tabel 1 Beknopte weergave van interactie-niveaus van ethanol en B(*a*)P en bevindingen van de hier beschreven studies
* bij mannetjesratten, ** bij vrouwtjesratten

De resultaten van ons onderzoek staan gecomprimeerd weergegeven in tabel 1 Deze figuur is een invulling van tabel 3 uit paragraaf 5 van de *General Introduction.*

We bestudeerden de invloed van blootstelling aan ethanol op de activering en inactivering van het carcinogene B(*a*)P, door het meten van excretieproducten, het meten van metabole activiteit in verschillende orgaanfracties en het bepalen van soort en hoeveelheid DNA-adducten. Niet alle denkbare mogelijkheden van interactie tussen ethanol en B(*a*)P zijn bestudeerd. Dit is aangegeven in figuur 1 (identiek aan figuur 6 uit de *General Introduction*)

De betekenis van onze observaties voor de effecten bij de mens moet nog worden vastgesteld Ons proefdieronderzoek leverde geen bewijs voor de hypothese dat het verhoogde kankerrisico van alcoholgebruikers te wijten is aan de verhoogde bioactivering van B(*a*)P door ethanol-inductie. Integendeel, de P450 iso-enzymen die belangrijk zijn bij het B(*a*)P-metabolisme zijn minder actief Een verandering in het fase II-metabolisme kan echter een rol spelen De gemeten vermindering in epoxide-hydrolase activiteit kan (mede) oorzaak zijn van de toegenomen hoeveelheden DNA-adducten in de lever van met ethanol behandelde ratten.

Epidemiologisch is een synergistisch effect aangetoond van alcohol- en tabaksconsumptie, met een toenemende kans op kanker van verscheidene organen als gevolg Verschillende additionele technieken en monsters, ook van menselijke oorsprong, zullen in *in vitro* en in *in vivo* onderzoek gebruikt moeten worden om het mechanisme van dit effect volledig op te helderen



Figuur 1 Schema van twee-staps carcinogenese en mogelijke aangrijpingspunten van ethanol (naar Seitz, 1988) Onderstreepte interactiemogelijkheden waren onderwerp van deze studie

PUBLICATIONS AND ABSTRACTS

Scientific publications

J.A.G. van de Wiel, C.M.P. Duijf, J.C.L.M. Pertijs, J.H.J. Copius Peereboom-Stegeman and R.P. Bos (1990) Growth and liver morphology after long-term ethanol consumption of rats. Laboratory Animals 24:265-272.

J.A.G. van de Wiel, M. Meuwissen, H. Kooy, P.H.S. Fijneman, J Noordhoek and R.P. Bos (1992) Influence of long-term ethanol treatment on in vitro biotransformation of benzo(*a*)pyrene in microsomes of the liver, lung and small intestine from male and female rats. Biochemical Pharmacology 44:1977-1984.

J.A.G. van de Wiel, P.H.S. Fijneman, C.M.P. Duijf, R.B.M. Anzion, J.L.G. Theuws and R.P. Bos (1993) Excretion of benzo(*a*)pyrene and metabolites in urine and feces of rats: Influence of route of administration, sex and long-term ethanol treatment. Toxicology 80:103-115.

J.A.G. van de Wiel, P.H.S. Fijneman, K.B. Teeuw, B. van Ommen, J. Noordhoek and R.P. Bos (1993) Influence of long-term ethanol treatment on rat liver biotransformation enzymes. Alcohol. *In press.*

J.A.G. van de Wiel, N. Ajubi, J.W.M. Gielen, M.-J.S.T. Steenwinkel, R.A. Baan and R.P. Bos (1993) Influence of long-term ethanol treatment on DNA-adduct formation in liver and intestinal epithelial cells of rats after one oral dose of benzo(*a*)pyrene. *Submitted.*

J.A.G. van de Wiel, Y. Jansma, J.J.A. Muller, E.D. Kroese and R.P. Bos (1993) Effect of long-term ethanol combined with short-term B(*a*)P treatment on DNA-adduct levels in several organs of rats. *Submitted.*

J.A.G. van de Wiel and W.F. Passchier (1993) Risk assessment: good for health? *Submitted.*

Abstracts

J.A.G. van de Wiel, C.P.M. Duijf, R.P. Bos, K.B. Teeuw, J.H.J. Copius Peereboom-Stegeman, J.C.L.M. Pertijs (1988) The influence of alcohol consumption on benzo(*a*)pyrene biotransformation in the rat. Proceedings of the XVIII Annual Meeting of the European Environmental Mutagen Society (EEMS). October 3-8, 1988, Varna, Bulgaria.

J.A.G. van de Wiel, C.P.M. Duijf, R.P. Bos (1989) The influence of alcoholconsumption on biotransformation in the rat. Annual meeting of the Dutch Society of Toxicology (NVT), January 11, 1989, Utrecht, Netherlands. Pharmaceutisch weekblad Scientific edition Supplement B, 11(2).

J.A.G. van de Wiel, J.W.M. Gielen, M.-J.S.T. Steenwinkel, R.A. Baan, R.P. Bos (1990) The influence of longterm ethanol exposure on the formation of DNA-adducts in rat liver and -gut after one oral dose of benzo(*a*)pyrene. Annual meeting of the Dutch Society of Toxicology (NVT), January 17, 1990, Utrecht, Netherlands.

J.A.G. van de Wiel, P.H.S. Fijneman, R.P. Bos (1990) B(*a*)P metabolites in urine of long-term ethanol pretreated rats. Proceedings of the VIIIth International Symposium on Microsomes and Drug Oxidations. Drug metabolizing enzymes: genetics, regulation and toxicology. M. Ingelman-Sundberg, J.-A. Gustafsson, S. Orrenius (eds.) June 25-29, 1990, Stockholm, Sweden.

J.A.G. van de Wiel, N. Ajubi, J.W.M. Gielen, M.-J. S. T. Steenwinkel, R.A. Baan, R.P. Bos (1991) The influence of long-term ethanol exposure on the formation of DNA-adducts in rat liver and -gut after one oral dose of benzo(*a*)pyrene. Proceedings of a symposium of the Dutch section of the European Environmental Mutagen Society. May 2, 1991, Bilthoven, Netherlands.

J.A.G. van de Wiel, P.H.S. Fijneman, J. Noordhoek, R.P. Bos (1991) Sex differences in benzo(*a*)pyrene toxicokinetics in the rat: the influence of chronic ethanol pretreatment. Proceedings of the Third international meeting of the International Society for the Study of Xenobiotics (ISSX). Drug metabolism: molecules, models and man. June 24-28, 1991, Amsterdam, Netherlands.

J.A.G. van de Wiel, P.H.S. Fijneman, J. Noordhoek, R.P. Bos (1991) Sex differences in benzo(*a*)pyrene toxicokinetics in the rat: the influence of chronic ethanol pretreatment. Proceedings of the joint meeting of the Dutch section and the Belgian section of the European Environmental Mutagen Society. November 30, 1991, Mol, Belgium.

J.A.G. van de Wiel (1992) De invloed van langdurige alcoholconsumptie op de toxicokinetiek van benzo(*a*)pyreen. Najaarsvergadering van de werkgroep *In vitro* Toxicologie van de NVT, 26 november 1991, Lelystad. TCDD 1992;15(1):10.

J.A.G. van de Wiel, W.F. Passchier (1993) Risk assessment: good for health?. Proceedings of the IVth European meeting of Environmental Hygiene. June 9-11, 1993, Wageningen, Netherlands.

Other publications

J.A.G. van de Wiel en E. van Hove (1985) Blaastest voor buikklachten. Technovisie; 15(7/8):6-7.

J.A.G. van de Wiel en E. van Hove (1985) Micro-organismen in de modder zetten oeroude samenleving voort. Technovisie;15(9):6-7.

J.A.G. van de Wiel en E. van Hove (1985) Revolutie in de evolutie. Technovisie;15(10):10-15.

J.A.G. van de Wiel (1985) Bot en biokeramiek. Technovisie;15(12):32-34.

J.A.G. van de Wiel (1987) Relatie tussen darmflora en darmkanker complex. Chemisch Magazine, bijvoegsel bij Chemisch Weekblad;23:417-420.

J.A.G. van de Wiel en R.P. Bos (1991) De toxische aspecten van alcohol. Giftig;8:20-25.

NAWOORD

Nederland was blijkbaar het laatste bolwerk waarin het schrijven van een proefschrift nog als levenswerk werd beschouwd (NRC Handelsblad, 9/9/'93). Binnen een niet al te nauw omschreven thema had de promovenda volop ruimte bij de invulling van het onderzoek. Met de komst van de AIO is het promoveren een meer gerationaliseerd studie-onderdeel geworden. Volgens sommigen terecht, maar ik vond het prettig het onderzoek voor een groot deel zelf in te vullen en het te combineren met zaken als het onderwijs aan studenten Gezondheids-wetenschappen. Ook naast mijn huidige baan bij de Gezondheidsraad is nog het nodige gelezen en geschreven over het onderwerp van dit boekje.

Het bedrijven van toxicologie is een fascinerende bezigheid. Aan de ene kant staat het abstracte modelmatige mechanismewerk achter de PC, aan de andere kant de onderbouwing van concrete maatregelen op het beleidsterrein van volksgezondheid en milieu.

Combinatietoxicologie staat volop in de belangstelling. Alleen al over de vraag hoe de realiteit van gelijktijdige blootstelling aan veel verschillende stoffen te benaderen, nee, óf deze realiteit wel meegewogen moet worden bij de humane risicoschatting lopen de meningen uiteen. Wetenschappelijk gezien was het in elk geval interessant een minuscuul onderdeel van het ons omringende interagerende stoffen-universum te bestuderen.

Voor de standaard voorbehandeling van de ratten met ethanol kon ik goed terecht bij de buren; het centrale proefdierlaboratorium van de Nijmeegse universiteit. Geert Poelen, Hennie Broekman, Theo van den Ing en Gerrie Grutters: bedankt voor de dagelijkse verzorging van de proefdieren, voor het uitprepareren van organen na behandeling met B(*a*)P in de toxunit en het catheteriseren ten behoeve van het online meten van bloedalcohol-waarden. Bij het methodologische hoofdstuk hoorde ook het bestuderen van leverslices onder de electronenmicroscoop. Doctor Copius Peereboom-Stegeman, beste Jenny, howel je wel eens meer 'interessante abnormaliteiten' zag dan mij lief was, moet men geprolifereerd SER toch ook eens met eigen ogen zien. Jeanne Pertijs maakt nog steeds de coupes en Arnold Meyer was de student die aan dit onderdeel het meest heeft bijgedragen.

Carla Duijf heeft in de aanloopfase van het onderzoek prima ondersteuning gegeven. Daarna werd Peter Fijneman een constante meewerkende factor. De prettige samenwerking met hem blijkt uit zijn co-auteurschap bij de meeste artikelen.

Een andere constante factor was natuurlijk doctor Bos. Beste Rob, je hebt een belangrijke rol gespeeld als bindmiddel voor de vakgroep in het interregnum. Hooggeleerde Henderson, beste Pie: jammer dat je al zo snel naar Maastricht vertrok. Hooggeleerde Noordhoek, beste Jan: jouw kritische blik, met name op het *in vitro* werk, was heel nuttig.

I would like to thank Dr. H.V. Gelboin of the Cancer Institute of the National Institutes of Health (Bethesda, MD, USA) for his stimulating suggestions at the Stockholm congress on Microsomes and Drug oxidations in Stockholm, 1990. Without his kind gift of several antibodies of the P450 isoenzymes, the work described in chapter IV would not have been possible.

Vier studenten hebben een belangrijke bijdrage geleverd aan het *in vitro* werk: de gezondheidswetenschappers Bart Teeuw en Marly Meuwissen, en de chemici Michel Elshof en Heimen Kooy.

Ook mensen van andere Universiteiten en onderzoeksinstituten in Nederland ben ik dankbaar voor hun morele of praktische ondersteuning. In Utrecht (RITOX) Bas Blaauboer, Heleen Wortelboer en Els de Groene (bij het tot stand komen van het hoofdstuk over enzymactiviteiten). In Zeist (ITV) Ben van Ommen (voor alle metingen aan glutathion-transferase). In Bilthoven (RIVM) Dinant Kroese en André Muller (bij het meten van DNA-adducten met de Phosphorimager). Ook de studente Yvonne Jansma heeft hier veel werk verzet. In Rijswijk (MBL-TNO) zijn Jacqueline Gielen en Nasser Ajubi een half jaar in de weer en in de leer geweest om DNA-adducten te bepalen met de postlabelling-techniek. Zij werden enthousiast ondersteund door Marie-José Steenwinkel. Rob Baan was hun externe begeleider.

Mijn huidige collega's bij de Gezondheidsraad toonden steeds hun belangstelling, en over combinatietoxicologie zijn we zeker nog niet uitgepraat.

Diverse familieleden, vrienden en bekenden zijn in het verleden gepromoveerd of werken nog aan hun thesis. Dat geeft herkenning en steun. De anderen ben ik juist erg dankbaar voor het vermijden van dit soort activiteiten.

De juiste doses relativering en stimulans, een snufje open-haard-gegenereerde PAKs, gevoegd bij een ethanolhoudend likeurtje; het vinden van het juiste recept om een promovenda op de been te houden, is traditioneel in de eerste plaats natuurlijk toebedacht aan de echtgenoot. Ingenieur doctorandus Nieuwesteeg, liefste Michaël.

Gouda, 9/9/'93

CURRICULUM VITAE

Jeanine van de Wiel, geboren op 25 februari 1960 te Eindhoven, behaalde in 1978 het diploma Atheneum-β aan het Eckart-College aldaar. In dat zelfde jaar begon zij aan de opleiding tot lerares biologie en scheikunde. In 1983 studeerde zij af aan het dr. Moller Instituut te Tilburg. Onderwerp van haar afstudeerscriptie was het gedrag van *Acinetobacter,* een voor de fosfaatverwijdering belangrijke bacterie uit in het actief slib van rioolwaterzuiveringsinrichtingen. Zij bracht hiervoor een half jaar door bij de vakgroepen Microbiologie en Waterzuivering van de Landbouwuniversiteit Wageningen.

In 1983 maakte zij de overstap naar de Katholieke Universiteit Nijmegen en 'deed' daar het hoofdvak microbiologie (*Metopus Striatus*, sapropel-organisme) bij prof. dr. G.D. Vogels. Andere doctoraalvakken waren populariseren van de natuurwetenschappen (prof. dr. J. Willems) en toxicologie (prof. dr. P.Th. Henderson). In deze jaren vervulde zij student-assistentschappen bij Microbiologie, Milieukunde en Toxicologie. Bovendien werkte zij als free-lance wetenschapsjournalist voor met name Technovisie, waarvan haar huidige echtgenoot toen hoofdredacteur was. In Tilburg was zij actief in de plaatselijke Werkgroep Natuurbehoud en Milieubeheer.

In 1987 studeerde Jeanine van de Wiel af aan de faculteit der Wiskunde en Natuurwetenschappen in Nijmegen. Een paar maanden eerder was zij als universitair docent aangesteld bij de vakgroep Toxicologie van de faculteit der Medische Wetenschappen. Daar werkte zij tot eind 1991 aan het in dit proefschrift beschreven onderzoek. Daarnaast ontwikkelde zij de onderwijsmodule Toxicologische Grenswaarden, Normen en Beleid en begeleidde studenten Gezondheidswetenschappen.

Sedert februari 1992 is Jeanine van de Wiel werkzaam als wetenschappelijk secretaris bij de Gezondheidsraad in Den Haag. Zij is ook secretaris van de landelijke stichting Vrouwen in Natuurwetenschappen, een professioneel deelnetwerk van de Stichting Vrouwennetwerk Nederland.

STELLINGEN

1 Over de wijze waarop de gezamenlijke toxiciteit van een groot aantal stoffen waaraan de mens tegelijkertijd blootstaat moet worden betrokken in de normstelling, bestaat nog steeds de onzekerheid die in 1985 is gesignaleerd in het advies van de Gezondheidsraad over uitgangspunten voor normstelling.

Gezondheidsraad Commissie Uitgangspunten voor normstelling Uitgangspunten voor normstelling de inzichtelijke opbouw van advieswaarden voor niet-mutagene, niet-carcinogene en niet-immunotoxische stoffen Den Haag Gezondheidsraad, 1985, publikatie nr 1985/31

2 Als men proefdieren wil blootstellen aan een in te nemen stof met een zekere calorische waarde, zoals ethanol, dient men een bewuste keuze te maken tussen een isonutriënt dieet of een isocalorisch dieet voor blootgestelde en niet-blootgestelde dieren en die keuze te vermelden in de beschrijving van de proefopzet.

International Agency for Research on Cancer Alcohol drinking Lyon, France, IARC monograph nr 44, 1988

3 Inductie van een specifiek iso-enzym van P450 (P4502E1) door ethanol in de lever van proefdieren, gaat samen met een verlaging van constitutieve P450 iso-enzymen.

Dit proefschrift

4 Blijkens de uitkomsten van onderzoek bij proefdieren is, in termen van de teratogene potentie, de dagelijkse inname van de 'reference dose' voor TCDD van de Environmental Protection Agency (6 fg per kg lichaamsgewicht) equivalent met de consumptie van één glas bier per 8000 jaar

B N Ames *et al* , Science 1978,236 271-280

B N Ames, Environmental and Molecular Mutagenesis 1989,14 66-77

5. De geldigheid van de stelling 'Medical research has been biased by taking the male as typical of the species - among humans as well as among animals' kan worden uitgebreid tot (vrijwel) alle vakgebieden.
The Economist, Science & Technology, 20/3/'93.

6. Door Seitz *et al.* is diverse malen een samengaan gerapporteerd van verhoogde activiteit van benzo(*a*)pyreen-hydroxylase en verhoogde vorming van mutagene B(*a*)P-metabolieten in lever- en dunne darmfracties van met ethanol behandelde proefdieren. De hierop door de onderzoekers gebaseerde verklaring van een toegenomen kankerrisico bij gecombineerde blootstelling aan alcohol en tabaksrook behoeft op z'n minst enige nuancering.
H.K. Seitz *et al*, Biochemical and Biophysical Research Communications 1978;85: 1061-1066
H K Seitz *et al.*, Cancer Letters 1981;13:97-102.

7. De gecombineerde blootstelling aan tabaksrook en alcohol, mits in de juiste sociaal-economische matrix, leidt slechts tot 'dancing fever'.
Fourth European ISSX meeting. Toxicological Evaluation of Chemical Interactions: Relevance of Social, Environmental and Occupational Factors Royal Hotel Carlton. July 3-6, 1992, Bologna, Italy.

Stellingen behorende bij het proefschrift:

The influence of ethanol on the biotransformation and genotoxicity of benzo(a)pyrene

Jeanine A.G. van de Wiel
Nijmegen, 17 november 1993